The role of the homeodomain protein Pitx3 in the development and survival of midbrain dopaminergic neurons.

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I declare that the work described in this Thesis is my own except where otherwise stated.

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Abbreviations

β-gal	β-galactosidase
αGSU	glycoprotein hormone α-subunit
AADC	L-aromatic amino acid decarboxylase
Aldh1	retinaldehyde dehydrogenase 1 (also known as AHD2)
ASMD	anterior segment mesenchymal dysgenesis
bHLH	basic helix-loop-helix
CNS	central nervous system
CPu	caudate putamen
D2R	D2 dopamine receptor
DA	dopaminergic
DAT	dopamine transporter
E	embryonic day
EB	embryoid body
eGFP	enhanced GFP
EGR1	early growth response 1
En	Engrailed
ER	estrogen receptor
ES	embryonic stem
Fgf	fibroblast growth factor
FSHβ	follicle-stimulating hormone β
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GH	growth hormone
GnRH	gonadotrophin-releasing hormone
HD	homeodomain
HygroTK	hygromycin-thymidine kinase
IL-1β	interleukin-1β
IRES	internal ribosomal entry site
IsO	isthmic organiser
mDA	midbrain dopaminergic neurons
MHB	mid-hindbrain boundary

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	nucleus accumbens
NTN	neurturin
OT	olfactory tubercle
P	postnatal day
pac	puromycin resistance gene
pCAG	CMV immediate early enhancer and chicken β-actin promoter
pCMV	cytomegalovirus immediate early-enhancer
PD	Parkinson's disease
PGK	phosphoglycerate kinase
POMC	pro-opio melanocortin
SA	splice acceptor
SDIA	stromal cell-derived inducing activity
SF1	steroidogenesis factor 1
Shh	Sonic hedgehog
SNc	substantia nigra pars compacta
STN	subthalamic nucleus
TGF-β_3	transforming growth factor-β_3
TH	tyrosine hydroxylase
TSH	thyroid stimulating hormone
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UCHL1	ubiquitin-C-hydrolase-L1
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area

Abstract

There is much interest in the study of midbrain dopaminergic (mDA) neurons as their functions include the regulation of motor function, emotion and reward pathways. Furthermore the dysfunction of these neurons is implicated in a number of human disorders such as Parkinson's disease (PD), addiction and schizophrenia. PD is characterised by the degeneration of mDA neurons of the substantia nigra pars compacta (SNc), therefore, research into the specification and development of mDA neurons is of particular interest in relation to this disease. An understanding of the development of mDA neurons may lead to new methods of preventing their degeneration or potentially a human ES cell derived source of mDA neurons that could be used for transplantation in PD patients.

Pitx3 is a bicoid-related homeodomain protein with an expression pattern restricted to the mDA neurons of the SNc and ventral tegmental area (VTA), within the central nervous system. To directly investigate a role for Pitx3 in mDA neuron development, I have analysed a line of transgenic mice with a green fluorescent protein (GFP) reporter under the control of the endogenous *Pitx3* promoter. Use of the targeted GFP reporter as a midbrain dopaminergic lineage marker in the phenotypically normal heterozygous mice identified previously unrecognised ontogenetically distinct subpopulations of dopaminergic cells within the ventral midbrain. These subpopulations were detectable at E12.5 based on their temporal and topographical expression of Pitx3 and TH.

Analysis of the *Pitx3* null mice revealed that Pitx3 is required for the survival of a subset of nascent mDA neurons at the beginning of their terminal differentiation. The loss of mDA neurons via apoptosis continued throughout development resulting in a complete absence of SNc neurons whilst the VTA remained relatively intact in adult *Pitx3* null mice. In addition, during embryonic development *Pitx3* deficiency caused a loss of tyrosine hydroxylase (TH) expression specifically in the SNc dopaminergic neurons. Analysis of chimeric mice made with *Pitx3* null and *Pitx3* heterozygous ES cells revealed that Pitx3 acts in a cell autonomous manner. These findings point to two roles for Pitx3 in SNc mDA neurons, one in their survival and the other in regulation of TH expression. Taken together, these studies suggest that the ontogenetically distinct subpopulations may provide the molecular basis for the specific dependence of substantia nigra DA neurons on Pitx3.

In addition, to establish whether the subpopulations identified at E12.5 do form the SNc and VTA, respectively, a strategy to track the fate of the earliest Pitx3-expressing cells has been initiated. In order to achieve this I have created transgenic mice in which a tamoxifen inducible form of Cre recombinase is under the control of the endogenous *Pitx3* promoter. These mice can be crossed with existing mice which contain a ubiquitously expressed Cre-inducible reporter, such as LacZ or GFP, to give a temporally and spatially restricted reporter expression.

Chapter 1

Introduction

1.1. Midbrain dopaminergic neurons

Dopamine containing neurons are present in various areas of the central nervous system (CNS) and are usually identified by the presence of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis. There are several groups of dopaminergic (DA) neurons in the CNS including the retina (A17), olfactory bulb (A16) and the diencephalic DA neurons (A11-14) (Bjorklund and Lindvall, 1984). However, the largest assembly of dopaminergic (DA) neurons in the brain are located in the ventral midbrain. The majority of the midbrain dopaminergic (mDA) neurons form functionally distinct subgroups called the substantia nigra pars compacta (SNc, A9) and the ventral tegmental area (VTA, A10) based on their position within the midbrain and the target structures which they innervate (Fig. 1.1) (Bjorklund and Lindvall, 1984). There is much interest in the mDA neurons as they are essential for important brain functions and their dysfunction/degeneration is associated with a number of disorders. Dopaminergic neurons of the SNc regulate motor function via nigro-striatal projections to the dorsolateral striatum and in humans the degeneration of SNc neurons results in Parkinson's disease (PD). PD affects 1% of the population aged over fifty, and is characterised by a slowness of movement (bradykinesia) and a difficulty in initiating movement (akinesia). On the other hand, mDA neurons of the VTA project to the ventromedial striatum, cortical areas and the limbic system forming the mesolimbocortical system. This system is involved in the modulation and control of cognitive, emotional and reward pathways (Kelley and Berridge, 2002). Defects of the mesolimbocortical system are implicated in addiction and psychiatric disorders such as depression and schizophrenia (Dailly et al., 2004; Sesack and Carr, 2002).

The current understanding of the specification and development of mDA neurons is limited. A number of factors involved have been identified, however, there are many gaps in the knowledge and the interactions between factors and the cascade of signalling events remain to be elucidated. Due to the important roles of mDA neurons in a number of human diseases it is of interest to fully understand the specification and development of these neurons. Furthermore, this knowledge is

necessary in order to realise the goal of creating pure populations of mDA neurons from embryonic stem (ES) cells in vitro. An ES cell-derived source of mDA neurons is desirable for a number of applications including pharmacological assays to analyse the activity of potential drug treatments for mDA system disorders and as a source of mDA neurons for transplantation studies in animal models of PD. The ultimate objective of the studies on the mouse ES cell system is to translate them to the human ES cell system, which may lead to the generation of human ES cell-derived mDA neurons for drug screening and potentially for transplantation into PD patients.

Both in vitro and in vivo studies will help to determine the key mediators involved in mDA neuron specification and development. In particular loss-of-function knockout studies are a powerful tool with which to elucidate the role of specific genes in embryonic mDA development. Although the in vitro system has more limitations, it is a convenient method for evaluating the effects of a variety of molecules, especially soluble factors, on undifferentiated cell populations.

1.2. In vitro mDA neuron specification

Since the exact requirements for differentiation of ES cells into specific cell lineages remains elusive, current differentiation protocols yield heterogeneous cell types. So far there have been two protocols developed specifically to differentiate ES cells towards a DA fate, one involves cell aggregation and the application of Shh and Fgf8 (Lee et al., 2000) and the other is via co-culture of ES cells with PA6 stromal cells (Kawasaki et al., 2000). In addition, a serum-free adherent monolayer culture method has been developed in which ES cells can develop into neural precursors, and subsequently DA neurons can be produced upon addition of Fgf8 and Shh (Ying et al., 2003).

Kawasaki and co-workers identified a stromal cell-derived inducing activity (SDIA) that efficiently induces neural differentiation from ES cells, with a high proportion of TH-positive neurons (Kawasaki et al., 2000). The nature of SDIA is unclear as PA6 neural inducing activity remains when the cells are fixed thus unable to secrete soluble factors, or when the PA6 cells are separated from the ES cells by a filter. The authors suggest that SDIA may be a secreted factor that is secondarily tethered to the cell surface, as treatment with heparin removes the neural inducing activity (Kawasaki et al., 2000). Recently it has been shown that the factors mediating SDIA

may be isolated in solution by treating PA6 cells with heparin and that the proliferative and neural differentiation effects of SDIA can be distinguished (Yamazoe et al., 2005). Using this method it was reported that TH-positive neurons appeared between days 6-8 of the induction period and constituted 30% of the total neurons in the culture (Kawasaki et al., 2000).

Another approach for DA fate induction from ES cells involves the addition of Shh and Fgf8 (Lee et al., 2000). This protocol starts with a short period of embryoid body (EB) differentiation followed by medium-based selection for nestin-positive neural precursor cells. The neural precursors are then expanded using Fgf2 before being induced to a DA fate by addition of Fgf8, Shh and ascorbic acid. The authors demonstrated that it was possible to obtain TH-expression in over 30% of the neurons (Lee et al., 2000). Using a similar approach, Rolletschek et al. use an array of survival-promoting factors in the production of DA neurons from ES cells (Rolletschek et al., 2001). They found that a cocktail containing interleukin-1β (IL-1β), glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), transforming growth factor-β_3 (TGF-β_3), and dibutyryl-cAMP (db-cAMP) could enhance RNA expression levels of *En1*, *Mash-1*, *dopamine receptor 2 (D_2R), TH* and *Nurr1* and increase the proportion of TH-positive and dopamine active transporter (DAT)-positive neurons from around 20% to around 40% (Rolletschek et al., 2001). However, it remains to be established as to the extent of those ES cell-derived DA neurons that express a midbrain phenotype.

An important caveat in many studies on mDA differentiation from ES cells is the use of markers in their analysis. Indeed, many *in vitro* studies assess mDA neuronal phenotype by determining TH expression in cultured cells. However, this has limitations as TH is expressed in all catecholaminergic neuronal types including adrenergic and noradrenergic neurons. Furthermore, other DA markers such as DAT, L-aromatic amino acid decarboxylase (AADC) and dopamine receptors are expressed in DA cells in other CNS regions including the olfactory bulb and the diencephalic DA cell groups located in the arcuate nucleus, periventricular nucleus, caudal thalamus, and hypothalamic regions (Bjorklund and Lindvall, 1984). Analysis of DA differentiation of *Pitx3*-GFP ES cells with the PA6 co-culture method revealed that the vast majority of *Pitx3*-GFP positive neurons express TH (Zhao et al., 2004). However, only around 10-15% of all TH or DAT expressing cells co-express *Pitx3*-GFP, suggesting that not all of the DA neurons generated by this method display a

mesencephalic character (Zhao et al., 2004). Therefore, ES cell-derived dopaminergic neurons in vitro are heterogeneous and do not all exhibit mesencephalic identity. This is significant as functional repair of PD appears to be specifically associated with mesencephalic grafts as demonstrated by transplantation experiments (Hudson et al., 1994).

1.3. Midbrain specification in vivo

Diverse neural cell types are generated in the nervous system from multipotent neural progenitor cells. Accordingly, the specification of cell fate is complex and is governed by the interplay of extrinsic and intrinsic signalling molecules, combined with cell-type and temporal specific factors. A grid-like set of positional identities is given to neural progenitors via gradients of signalling molecules secreted throughout the dorsal-ventral and rostral-caudal axes of the neural tube. The floor plate and roof plate along the neural tube act as inducing centres and in addition, secondary organisers act as local signalling centres to further refine cell fates at the rostral-caudal level. Once neuronal fate has been restricted by these extrinsic cues, intrinsic signals, often transcription factors direct further differentiation into a mature, postmitotic neuron (Edlund and Jessell, 1999; Lumsden and Krumlauf, 1996; Stern, 2001)

During embryogenesis mDA neurons are specified in the ventral mesencephalon just rostral to the isthmic organiser (IsO) and in close proximity to the floor plate (Fig. 1.2). The IsO is a signalling centre located at the border between the midbrain and the hindbrain (the mid-hindbrain boundary, MHB) and its position determines the location and size of the mDA neuron population (Brodski et al., 2003). Accordingly, the correct specification of the IsO is necessary for the development of midbrain structures. Many molecules involved in establishing and patterning the midbrain are expressed in a complicated network of regulation in the isthmic region, which has not yet been fully uncovered. At the end of gastrulation, embryonic day (E) 7.5 in mouse, the future position of the IsO is identified where the expression domains of the transcription factors Otx2 and Gbx2 meet. Otx2 is expressed on the rostral side and Gbx2 is expressed on the caudal side of the IsO, and they are required to suppress hindbrain and midbrain development respectively (Li and Joyner, 2001). Also expressed from this stage is the homeobox transcription factor Lmx1b, which is initially expressed throughout the ventral mesencephalon and diencephalon and is later restricted to post-mitotic mDA neurons (Smidt et al.,

2000). At E8, the transcription factor Pax2 is expressed across the Otx2/Gbx2 border and expression of the secreted molecule Wnt1 is predominantly limited to the Otx2 domain (Wurst and Bally-Cuif, 2001) (Fig. 1.2). Following this, the transcription factors En1, En2 and Pax5 are expressed across the Otx2/Gbx2 border, and fibroblast growth factor 8 (Fgf8) is expressed on the Gbx2-positive side (Wurst and Bally-Cuif, 2001). By E9.5 the expression of Wnt1 and Fgf8 domains have sharpened into rings on rostral and caudal sides of the IsO, respectively (Wurst and Bally-Cuif, 2001). Otx, Lmx1b and Wnt1 are expressed on the midbrain side of the IsO whilst Gbx2 and Fgf8 are expressed on the hindbrain side.

1.4. Factors involved in mDA neuron specification and development

The correct specification and development of mDA neurons depends upon a number of extrinsic and intrinsic signalling factors which interact to create a specific identity code for mDA neurons. The majority of studies report the first mDA neurons detected by the presence of TH at E11.5 (Foster et al., 1988; Solberg et al., 1993; Zetterstrom et al., 1997), however TH expression in the mesencephalon as early as E9.5 has been reported (Di Porzio et al., 1990). The mechanisms involved in the regulation of TH expression in mDA neurons remain unclear. It appears, however, that TH is not required for mDA development, as *TH*-deficient mice have an apparently intact mDA system with normal projections to target tissues in the telencephalon (Zhou and Palmiter, 1995). Prior to TH expression the earliest known marker for mDA neuron progenitors is retinaldehyde dehydrogenase 1 (Aldh1, also known as AHD2), which is expressed at E9.5 in mDA progenitors and expression continues in mDA neurons throughout development (Wallen et al., 1999). The function of Aldh1 is to oxidise retinaldehyde into retinoic acid, however, a possible role for Aldh1 in mDA neuron development has not been investigated. Subsequently the first post-mitotic mDA precursors can be detected at around E10.5 identified by the expression of Nurr1 (see below). Studies using autoradiographic methods in the developing rat brain have demonstrated that most post-mitotic cells of the mDA system emerge between E11 and E15 (approximately equivalent to E10-E14 in mouse), with a peak of SNc neurons emerging at E13-E14 (E12-E13 in mouse) and VTA neurons at E14-E15 (E13-E14 in mouse) (Altman and Bayer, 1981; Hanaway et al., 1971; Lauder and Bloom, 1974). Some extrinsic and intrinsic factors that are implicated in mDA specification and development are discussed below.

1.4.1. Shh and Fgf8

The intersection of the floor plate-derived signal Sonic hedgehog (Shh) with the isthmus-derived Fgf8 creates an inductive centre for mDA neurons just rostral to the IsO (Fig. 1.2). It has been shown that Shh is necessary and sufficient for the induction of mDA neurons along the dorso-ventral axis, whereas Fgf8 is responsible for the position of mDA neurons along the anterior-posterior axis of the neural tube (Ye et al., 1998). However, recent studies have highlighted that the mDA-inducing effects of Shh and Fgf8 are complex and that interactions with other factors are required for correct cell-type specification in the midbrain. It was shown that as well as determining the rostral-caudal position of Fgf8 signalling (Ye et al., 2001), Otx genes also control Shh distribution along the dorsal-ventral axis in a dose-dependent repressive manner (Puelles et al., 2003). Furthermore Otx2 has a role in the formation and maintenance of ventral midbrain progenitor domains as defined by the expression pattern of Shh and the Shh-responsive class II homeobox transcription factors Nkx6.1 and Nkx2.2. Selective disruption of Otx2 expression in the ventral and lateral midbrain results in a considerable reduction in mDA neurons and an induction of progenitors with a rostral hindbrain identity, even though Fgf8 expression remains intact (Puelles et al., 2004). However, recent fate mapping studies have shown that mDA precursors are only responsive to Shh between E7.75 and E9 and beyond this stage mDA neurons are generated from non-Shh responsive precursors, thus they are specified independent of Shh signalling. The subtype and the number of mDA neurons formed from the Shh-responsive cells remains unclear (Zervas et al., 2004).

1.4.2. Wnt

The Wnt family of glycoproteins are signalling molecules with key roles in regulation of patterning, cell proliferation and cell determination in the embryo. Furthermore, within the nervous system Wnt proteins are involved in virtually all significant patterning events (Patapoutian and Reichardt, 2000). Wnt1 is expressed around the IsO, predominantly on the midbrain side. A key role for Wnt1 in midbrain development was demonstrated by loss of function studies which revealed a failure by *Wnt1*-deficient embryos to develop any midbrain or anterior hindbrain structures (McMahon and Bradley, 1990; McMahon et al., 1992). Later, complementary misexpression studies in the chick demonstrated a role for Wnt1 in the formation

and maintenance of the IsO. In this study, Wnt1 was shown to be able to induce Fgf8 expression, which in turn induces the expression of Lmx1b (Matsunaga et al., 2002). More recently fate mapping studies have revealed that cells in the mesencephalon expressing Wnt1 from E7.75 to E11.5 give rise to mDA neurons (Zervas et al., 2004). Therefore, Wnt1 may have a significant role in the specification of mDA neurons.

In addition to Wnt1, Wnt5a is also expressed at a high level in the ventral midbrain, with a peak at E11.5 in rat (Castelo-Branco et al., 2003). The addition of Wnt5a to rat E14.5 ventral midbrain primary cultures resulted in an increase in the number of TH-expressing neurons. Furthermore, there was an increase in the overall expression levels of *Pitx3* and *c-ret* in cultures treated with exogenous Wnt5a. From these studies the authors suggested that Wnt5a increases the total number of mDA neurons by promoting the acquisition of mDA phenotype, whilst Wnt1 acts as a general midbrain mitogen (Castelo-Branco et al., 2003).

1.4.3. Nurr1 (Nr4a2)

Nurr1 (also known as Nr4a2) is a member of the orphan nuclear receptor family of transcription factors and is expressed in the ventral mesencephalic flexure from mouse E10.5 onwards (Zetterstrom et al., 1997). In addition, Nurr1 is also expressed in other brain regions including neocortex, hypothalamus, hippocampus and cerebellum (Zetterstrom et al., 1996). Nurr1 deficient mice lack TH-expression in the ventral midbrain from E11.5, other DA neuronal markers including the retinoic acid converting enzyme Aldh1, c-ret and the D2R are also absent from the adult SNc and VTA (Zetterstrom et al., 1997). Further studies have revealed that *Nurr1* deficient neuroepithelial cells undergo normal ventralisation and differentiate into Pitx3 expressing neurons (Saucedo-Cardenas et al., 1998). The expression of En1, En2, Aldh1 and AADC has also been demonstrated in midbrain cells of E11.5 *Nurr1* mutant mice, although the expression of these markers is reduced or absent by E15.5 (Smits et al., 2003; Wallen et al., 1999). These early *Nurr1* deficient midbrain neurons do not express DA neuron markers such as TH and vesicular monoamine transporter 2 (VMAT2) (Smits et al., 2003) and subsequently die by apoptosis resulting in a severe loss of SNc and VTA cells by neonate stage. In addition the mDA markers that were expressed in *Nurr1* mutant mice by E15.5 are medially located. A further investigation into the distribution of the mutant *Nurr1* mRNA expression in *Nurr1* null brains revealed that *Nurr1* null mDA neurons fail to migrate laterally to their normal positions during development and are unable to innervate

their target tissue, the striatum (Wallen et al., 1999). Thus, Nurr1 is required for specific features of the dopaminergic phenotype, is necessary for the survival of late dopaminergic neurons and may also have a role in the migration of mDA neurons.

1.4.4. Engrailed

Engrailed is a homeodomain transcription factor that has two vertebrate homologues, En1 and En2. Both En1 and En2 are expressed in the developing mouse brain from E8 on either side of the IsO in the midbrain and hindbrain (Davis and Joyner, 1988). In adulthood En1 and En2 have a more limited expression pattern. In the midbrain, En1 is expressed throughout the SNc and VTA at high levels, whereas En2 is expressed at lower levels and is restricted to a subset of the En1 expressing cells (Simon et al., 2001). In addition, En1 and En2 are expressed in motor nuclei of the pons and En2 is expressed in the cerebellum (Davis and Joyner, 1988). *En1* null mice have major brain defects during embryonic development, including deficits in the midbrain, hindbrain, colliculi, cerebellum and the third and fourth cranial nerves (Wurst et al., 1994). Yet, mDA neurons are present in *En1* null mice at P0, and En2 expression is up-regulated in these cells (Simon et al., 2001). The *En2* knockout phenotype is milder with no gross mid-hindbrain abnormalities (Joyner et al., 1991). In contrast, the compound mutation of *En1* and *En2* results in a complete absence of both SNc and VTA DA neurons by E14. At E12 however, TH-positive neurons are present in the ventral midbrain of *En1/2* double mutants although there are fewer cells than in the wild type controls. At E13 the number of TH-positive cells is more reduced in the *En1/2* double mutants and some of the remaining TH-positive cells display signs of apoptosis (Alberi et al., 2004). However, there is a substantial truncation of the midbrain and anterior hindbrain in the engrailed double mutants resulting in a loss of the isthmus and reduced levels of Shh and Fgf8 (Simon et al., 2001). Accordingly it is difficult to make assumptions about the function of En1 and En2 in mDA neuron development in a situation where key midbrain structures and developmental signalling factors are aberrant. However, a recent study has addressed this by performing primary culture experiments with E12 En1/2 double mutant midbrain cells. This revealed that the requirement for En1 and En2 is cell autonomous and is independent of the mDA neuron environment. It was shown that mDA neurons lacking En1 and En2 died via apoptosis in vivo and in primary culture (Alberi et al., 2004). Thus the engrailed genes appear not to be required for mDA neuron specification but are required for their survival.

1.4.5. Lmx1b

Lmx1b is a LIM class homeobox (LIM-HD) gene that is expressed in the CNS and in the periphery where it is necessary for dorsoventral patterning of limbs (Riddle et al., 1995). During development Lmx1b is expressed in many areas of the CNS including those that give rise to SNc, VTA, Raphe nuclei, subthalamic nucleus (STN), posterior hypothalamus and the spinal cord (Asbreuk et al., 2002). Recently it has been reported that Lmx1b is required for serotonergic neuron development in the hindbrain (Ding et al., 2003). Lmx1b is expressed throughout the mesencephalon and diencephalon from E7.5 in mice and expression in the SNc and VTA has been reported from E12 onwards (Nunes et al., 2003; Smidt et al., 2000). Studies on *Lmx1b* null mice have revealed that a loss of Lmx1b results in the failure of proper mDA neuron development. At E12.5 there is a reduction in the number of TH-expressing cells and an absence of Pitx3 expression in the ventral midbrain of *Lmx1b* null mice. However, Nurr1 expression in the ventral midbrain is intact in *Lmx1b* null mice at E12.5. From E16 onwards, TH expression is not detected in the mutant ventral midbrain (Smidt et al., 2000). As Lmx1b is expressed early (E7.5 in mouse) during CNS patterning events and has a widespread expression pattern in the brain, it is difficult to draw conclusions about a direct role for Lmx1b in mDA neurons from this study. Further experiments are needed to address the cell autonomous functions of Lmx1b in mDA neurons. However, the knockout study suggests that Nurr1 is not regulated by Lmx1b and that Pitx3 might be a target of Lmx1b.

1.5. Pitx3

Pitx3 was identified by two groups at approximately the same time: Semina *et al* (1997) aimed to further characterise the *Pitx* family by screening a mouse cDNA library with a probe comprising the *Pitx2* HD, which led to the identification of *Pitx3*. Expression studies demonstrated that *Pitx3* mRNA was expressed in the lens of the eye from E11. *Pitx3* was mapped on mouse chromosome 19 close to *aphakia*. Also, distinct mutations in the human homologue of the mouse Pitx3 gene have been identified in patients with cataracts and anterior segment mesenchymal dysgenesis (ASMD) (Semina et al., 1998). On the other hand, Smidt *et al* (1997) identified Pitx3 while searching for homeobox genes associated with a unique neuronal lineage. Using in situ hybridisation they demonstrated that within the CNS *Pitx3* is exclusively expressed in the SNc and VTA and is superimposed with TH expression. In developing mice *Pitx3* mRNA expression is first detected in the

midbrain at E11.5 as a small layer at the ventral surface of the mesencephalic flexure. This layer corresponds to the first TH-expressing cells in the mouse brain. In situ hybridisation studies on human brains revealed that Pitx3 was expressed in pigmented substantia nigra neurons and there were fewer Pitx3-expressing cells in the substantia nigra of patients with PD (Smidt et al., 1997). As well as the eyes and the midbrain Pitx3 expression was also reported in the tongue, incisor primordia, mesenchyme around the sternum and vertebrae and in the head muscles (Semina et al., 1998; Smidt et al., 1997).

So far few potential Pitx3 targets and/or co-factors have been identified. A role for Pitx3 in the induction of TH expression has been suggested by the demonstration that Pitx3 can bind to response elements and activate the TH promoter in a cell type-dependent manner (Cazorla et al., 2000; Lebel et al., 2001). However, overexpression of Pitx3 in adult hippocampus-derived progenitor cells had no effect on the expression of DA neuron markers including the dopamine synthesising enzymes TH and AADC, the neurotrophic factor GDNF, the signal transducing receptors c-ret and GFRalpha-1 and the D2 dopamine receptor (D2R) (Sakurada et al., 1999). This implies that, unlike Nurr1, which can induce a DA phenotype in both neuronal and non-neuronal cells (Sonntag et al., 2004), Pitx3 may require cell type specific factor(s) to regulate TH expression. A potential Pitx3 co-factor may be Nurr1, as it has been suggested that Pitx3 and Nurr1 cooperate to regulate TH expression, and that Pitx3 may be involved in the maintenance of TH expression (Cazorla et al., 2000).

1.6. *Pitx* gene family

Homeobox genes encode transcription factors that have been shown to mediate key processes in development and patterning. The Pitx proteins belong to the *bicoid*-related subclass of *paired* homeodomain proteins, as they have a lysine at residue 50 in the homeodomain region (position 9 in the recognition helix of the homeodomain), which determines the DNA binding specificity of these proteins. Few *bicoid*-related homeodomain genes have been identified in mammals: they include *goosecoid* and the *Otx1* and *Otx2* genes. Goosecoid is expressed by organizer cells and the anterior visceral endoderm in the vertebrate embryo, and it has been proposed that it plays a role in the regulation of neural inducing activity (Sawada et al., 2000; Zhu et al., 1999). Otx1 has multiple roles in the developing and adult mouse, and is required for correct brain and sense organ development. Otx2 plays a

key role in anterior neural patterning and is required for the correct positioning of the isthmic organiser and mDA neuron specification (Acampora et al., 2001; Puelles et al., 2004).

Three members of the Pitx family have been identified: Pitx1, Pitx2 and Pitx3. They share a high amino acid sequence homology, for example, in mouse the homeodomains of Pitx2 and Pitx3 are identical and the homeodomain of Pitx1 has 96% homology to this (Fig. 1.3). The C-terminal domains of Pitx1, Pitx2 are around 50% conserved relative to Pitx3, however Pitx1 and Pitx2 isoforms have 70% amino acid identity. In addition there is a conserved 14 amino acid region at the C-terminus known as the OAR sequence which is present in several homeodomain genes. It has been shown that the C-terminal of Pitx2 containing the OAR region functions as an inhibitor of DNA binding activity and involved in protein-protein interactions (Amendt et al., 1999). Similarly, studies on Pitx2 have revealed that the N-terminal region is also important for regulating Pitx2 transcriptional activity (Amendt et al., 1999), however, the N-terminal domains of the Pitx family members are largely disparate.

1.6.1. Pitx1

Pitx1 was identified as a transcription factor involved in activation of pituitary-specific gene transcription of pro-opio melanocortin (POMC) (Lamonerie et al., 1996). As well as the pituitary, Pitx1 expression has been detected in the stomodeum, the first branchial arch mesenchyme, oral ectoderm and lateral plate mesoderm (Lanctot et al., 1997). In the pituitary, Pitx1 binds a promoter regulatory element with a sequence related to the Drosophila bicoid binding sequence, and acts in synergy with other cell-restricted factors to activate the POMC gene (Drouin et al., 1998; Lamonerie et al., 1996). Additionally, recent studies have revealed that Pitx1 may act in a positive auto-regulatory manner dependent on its DNA-binding and trans-activation domains (Goodyer et al., 2003). Other roles that have been attributed to Pitx1 include early hindlimb bud formation (Marcil et al., 2003). More recently Pitx1 has been shown to have a role as a tumor-suppressor gene in human cells, functioning to down-regulate the RAS pathway via direct transcriptional activation of RASAL1 (Kolfschoten et al., 2005).

1.6.2. Pitx2

Pitx2 was identified by positional cloning of the Rieger syndrome gene in humans. Mutations in one allele *Pitx2* may cause Rieger syndrome, which is characterised by abnormalities of the anterior chamber of the eye, dental hypoplasia, craniofacial

dysmorphism and iris hypoplasia (Semina et al., 1997). Similarly in mice there is a Pitx2 haploinsufficiency defect which recapitulates features analogous to human Rieger syndrome (Gage et al., 1999b). Major sites of Pitx2 expression in mice include eye, pituitary, forelimb and hindlimb mesenchyme, teeth, heart, forebrain and midbrain (Gage et al., 1999a). The complete loss of Pitx2 is lethal by E14.5 due to profound heart defects; also there are major abnormalities in the eyes, teeth, pituitary and lungs (Gage et al., 1999b; Kitamura et al., 1999; Lin et al., 1999). Roles for Pitx2 have been demonstrate in anterior structure formation (Gage and Camper, 1997), hindlimb bud development (Marcil et al., 2003) and multiple stages of pituitary development, where it acts in synergy with Pitx1 (Suh et al., 2002). A role for Pitx2 in vertebrate patterning of laterality has been demonstrated, as it appears to be the most downstream gene in the Shh/nodal/lefty-2 pathway controlling left-right patterning (Logan et al., 1998; Piedra et al., 1998; Yoshioka et al., 1998). Additionally, it has been proposed that Pitx2 has a role as a regulator of gamma-aminobutyric acid (GABA)-ergic neuron differentiation during mammalian neural development. Pitx2 is an ortholog of unc-30, a *C.elegans* gene that directly regulates glutamic acid decarboxylase (GAD, enzyme required for GABA biosynthesis) synthesis and has a central role in the specification of the GABAergic neurotransmitter phenotype. Pitx2 is expressed in regions where GABAergic neurogenesis occurs thus it may have a role in regulating GAD expression in mammals (Westmoreland et al., 2001).

1.7. Hypotheses and aims

Based on the restricted expression pattern of Pitx3 in mDA neurons combined with the fact that Pitx3 is a homeodomain transcription factor, which are typically involved in regulating development, the hypothesis was formed that Pitx3 may have a role in the development of mDA neurons. Therefore, the aim of this study is to investigate the role, if any, of Pitx3 in mDA neurons. Firstly the expression of Pitx3 in mDA neurons in mouse embryo and adult will be assessed to confirm the restriction of Pitx3 expression to mDA neurons within the CNS and the expression in both VTA and SNc neurons. Then, in order to understand the functional significance of Pitx3 in vivo the phenotype of mice that lack Pitx3 will be analysed.

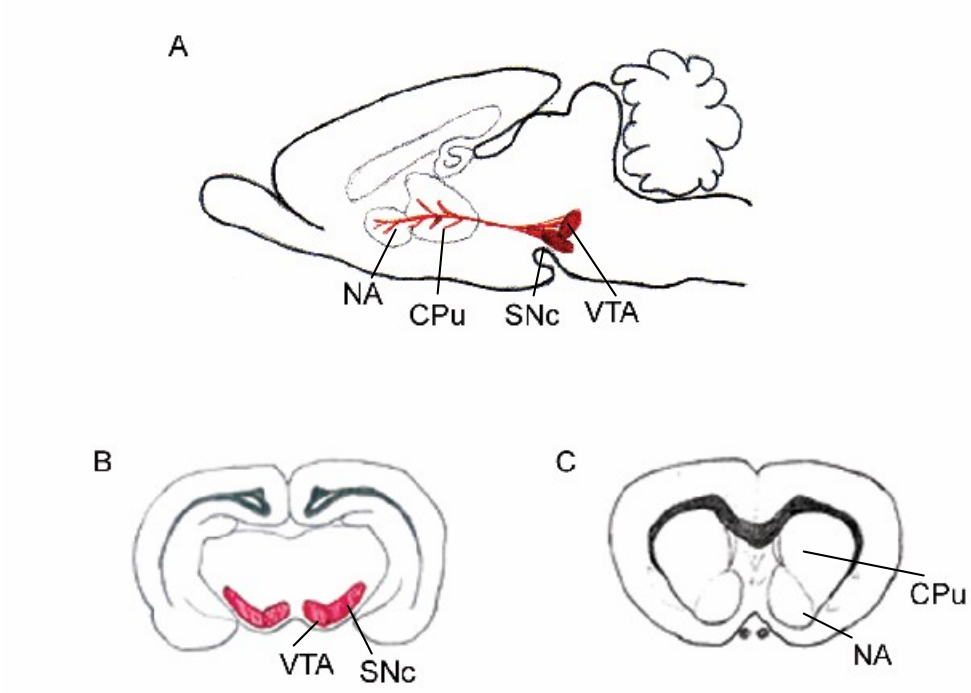

Figure 1.1. Schematic diagram showing the anatomy of midbrain dopaminergic neurons.

Diagrams of coronal sections through the midbrain (A) and the forebrain (B) show the location of the SNc and VTA neurons in the midbrain and their target areas of the striatum which are the dorso-lateral caudate putamen (CPu) for the SNc and the ventral CPu, nucleus accumbens (NA) and olfactory tubercle (OT) for the VTA.

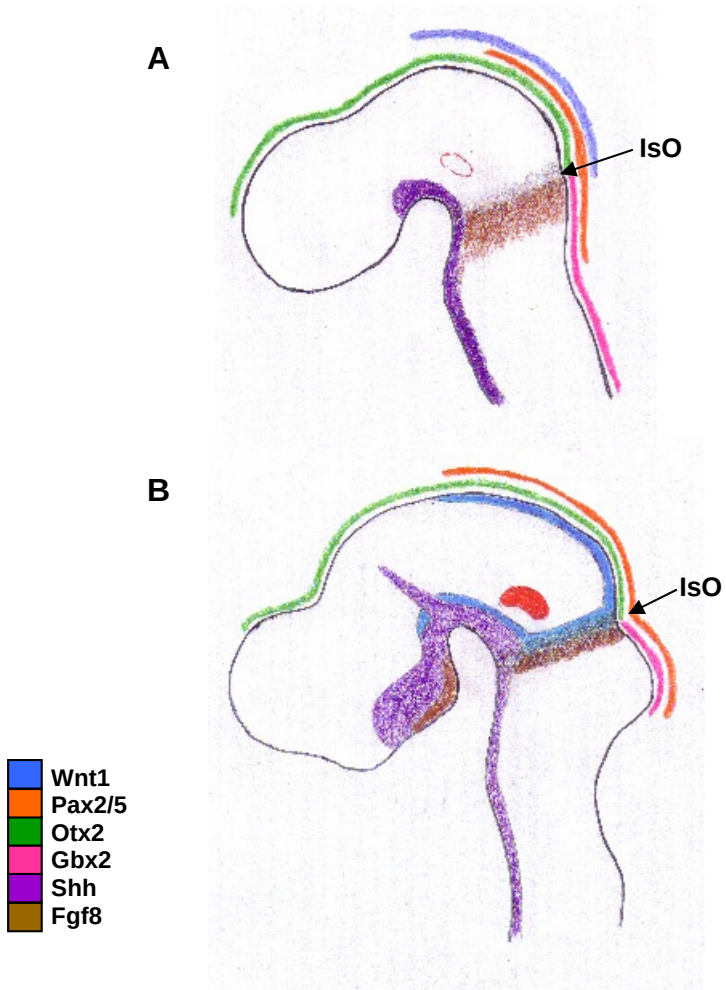

Figure 1.2. Schematic diagram showing position of mDA neuron specification and expression of genes during embryonic development.

The diagrams show a sagittal view of mouse neural tube at around E8 (A) and E11 (B) with the expression pattern of some genes involved in midbrain and mDA neuron development. At E8 (A) the expression domains of Otx2 and Gbx2 meet at the midbrain/hindbrain boundary (Isthmic organiser, IsO). The expression of Wnt1, Pax2, Pax5 and Otx2 overlaps in the midbrain region and Fgf8 is expressed on the caudal side of the IsO. The position where the mDA neurons will first appear is outlined in red (adapted from (Simon et al., 2003)). At E11 (B) the expression of Wnt1 and Fgf8 is restricted to narrow rings on either side of the IsO. The expression domain of Pax2/5 has also become narrower. The mDA neurons (red) are generated in the ventral midbrain just rostral to the IsO (adapted from (Wurst and Bally-Cuif, 2001)).

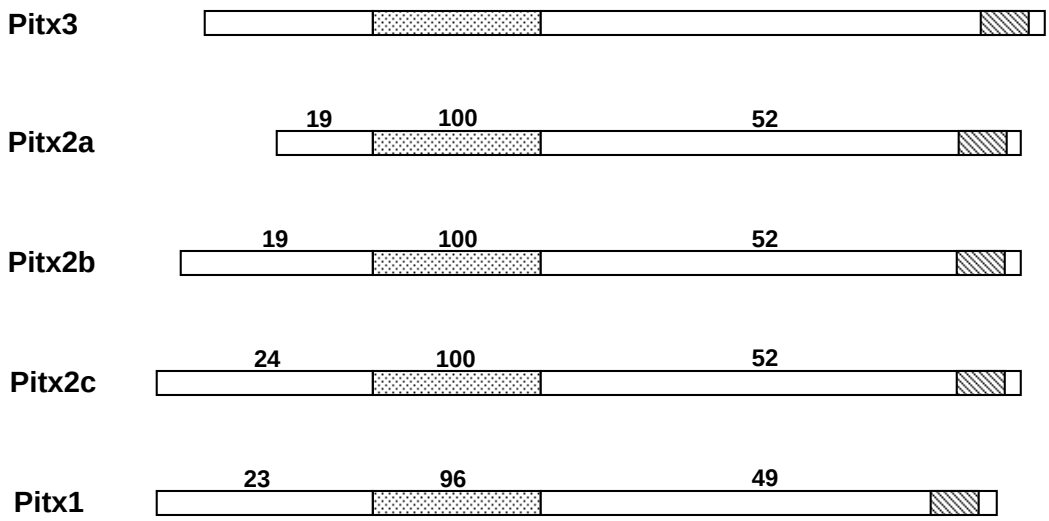

Figure 1.3. Diagram showing structure and amino acid identity between Pitx family genes.

The diagram shows the percent amino acid identity of Pitx family genes relative to Pitx3 within the homeodomains (spotted) and the regions N- and C-terminal to the homeodomain. The C-terminal OAR sequence is also indicated (striped) and it is fully conserved between Pitx3 and all Pitx2 isoforms, whereas the Pitx1 OAR sequence has 92% amino acid identity with Pitx3.

Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise stated analytical grade chemicals were obtained from either Sigma or BDH Laboratory Supplies (Merc Ltd). Stock solutions were prepared with reverse osmosis purified (ROP) water and filtered or autoclaved as necessary. Agarose for electrophoresis was supplied by Invitrogen. Synthetic oligonucleotides were synthesised by SIGMA. Radiosotypes were supplied by Amersham Biosciences.

2.1.1. Solutions

Phosphate buffered saline (PBS):
2.7 mM KCl, 137 mM NaCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4 (pH 7.4).

PBST:
0.2% (v/v) Triton X-100 (BDH) and 1% (w/v) bovine serum albumin (BSA) in PBS.

1X TAE:
0.04M Tris-acetate, 0.001M EDTA

20X SSC:
3M NaCl, 0.3M tri-Na citrate

Lysis buffer:
10mM Tris (pH 8.0), 50mM EDTA, 100mM NaCl, 0.5% SDS, 0.5mg/ml Proteinase K (Roche) added just before use.

Glasgow minimum essential medium (GMEM):
1X GMEM (Sigma), 10% (v/v) fetal bovine serum (Gibco), 1mM sodium pyruvate (Gibco), 1X MEM non-essential amino acids (Gibco), 2mM l-glutamine (Gibco), 0.1mM 2-mercaptoethanol (BDH).

Luria-Bertani (LB) broth:

1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract, 85mM NaCl.

LB agar:
1.5% (w/v) agar (Difco) in LB broth.

MOPS (10X):
0.2M MOPS (pH 7.0)
20mM sodium acetate
10mM EDTA (pH 8.0)

TFB1:
30mM potassium acetate, 10mM CaCl2, 50mM RbCl,, 15% glycerol, pH adjusted to 5.8 with 1M acetic acid and filter sterilised.

TFB2:
10mM MOPS, 75mM CaCl2, 10mM RbCl, 15% glycerol, pH adjusted to 6.5 with 1M KOH and filter sterilised.

Depurination solution:
11ml HCl, 989ml dH2O

Denaturation solution:
87.66g NaCl, 0.4M NaOH. Make up to 1L with distilled H_2O.

Church & Gilbert hybridisation buffer:
0.5M Na_2HPO_4 (pH 7.2), 70g/L SDS, 1mM EDTA.

Southern blot prehybridisation/hybridisation solution:
0.2mg/ml salmon sperm DNA (denatured by boiling in a waterbath for 10 mins), 1mM NaOH, 4.5mM Tris HCl pH 8.0 in Church & Gilbert hybridisation buffer at 65ºC.

STE buffer:
10mM Tris-Cl (pH 8.0), 0.1M NaCl, 1M EDTA (pH 8.0).

2.2 Animal analysis

Mice were housed and bred in the Biomedical Unit within the Institute for Stem Cell Research, according to the Animals (Scientific Procedures) Act (UK) 1986. Mice were kept in a 12 hours dark, 12 hours light cycle with the midpoint of the dark cycle at 1am. For collection of embryos, matings were set up and female mice were checked daily before 10am for the presence of a vaginal plug and if present then 1pm on that day was designated as embryonic day (E) 0.5. Mice aged 6 weeks or older were used for mating. Litters were left with their mother until 3 weeks of age when they were weaned by separating the offspring from the parent. At weaning the mice were sexed and tail tips were taken for genotyping. Maintenance of the mice, breeding set-up and tail biopsies were performed by the Biomedical Unit staff at the Institute for Stem Cell Research.

2.2.1. Vibratome sectioning

Embryos were dissected in PBS, then fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4°C overnight (for E12.5 and older) or 4 hours (E10.5 to E11.5), before embedding embryos in 2% agarose. Embryos were then cut on a vibratome at 100µm and mounted on slides. A Leica MZ FLIII Fluo Combi Stereo microscope and Openlab software were used to collect images.

2.2.2. Cryostat sectioning

Embryos and adult brains were dissected in PBS, then fixed in 4% PFA in PBS (pH 7.4) overnight for E12.5 and E14.5 or 4 days for adult brains. The embryos or brains were then cryoprotected by placing them in a solution of 30% sucrose in PBS, which was left at 4°C until the embryo or brain sinks. Embryos and brains were then embedded in OCT (BDH) in foil parcels over dry ice and stored at -80°C until use. Cryostat sectioning was performed at -16 to -19°C using a Leica CM 1900 cryostat and sections were cut at 20µm. Sections were collected on polysine slides (BDH) and allowed to dry at room temperature for 30 minutes and then were either used immediately or stored at -80°C until use.

2.2.3. Wax sectioning

Brains from newborn mice were washed twice in PBS and dehydrated through an ethanol series (1 wash 70% ethanol, 1 wash 95% ethanol, 3 washes 100% ethanol for 1 hour each wash) followed by clearing in xylene overnight. Embryos were then incubated through 3 washes of wax at 60°C for 1 hour each before embedding in wax. Brains were sectioned at 7µm on a microtome (Anglia Scientific) and collected

on polysine slides (BDH). Sections were dewaxed in xylene and then processed for immunohistochemistry or histological staining.

2.2.4. Immunohistochemistry

Frozen sections were allowed to equilibriate to room temperature. Sections were blocked with 3% normal serum in PBST for 1 hour at room temperature. Prior to the blocking step, sections to be visualised with DAB were incubated in 3% H_2O_2 in methanol for 15 minutes at 4°C to quench endogenous peroxidase activity. Then sections were incubated with primary antibodies in blocking solution at 4°C overnight in a humidified box. Sections were washed three times for 20 minutes each in PBST, then incubated with horse radish peroxidase (HRP)- or fluorescence-conjugated secondary antibodies at room temperature for 1 hour or overnight at 4°C, respectively. Sections to be visualised with DAB were incubated with biotinylated anti-rabbit IgG (Vectastain Elite, ABC kit; Vector Labs) for 1 hour at room temperature followed by avidin-biotinylated-peroxidase complex for 1 hour at room temperature, then DAB substrate until colour developed. After washing three times with PBS, cell/sections were mounted in Immunofluore (ICN Biomedicals) and analyzed using a Zeiss Axiophot microscope or Leica confocal microscope.

2.2.4.1. Antibodies

Primary antibodies:

Antigen	Species	Supplier	Concentration
βtubulin3	mouse	Babco	1:500
Caspase-3 active	rabbit	R&D systems	1:1000
Engrailed 1	rabbit	Developmental Studies Hybridoma Bank	1:100
GAD	rabbit	Sigma	1:3000
GFP	chicken	Chemicon	1:1000
Ki67	rabbit	Novocastra	1:500
Pitx3	rabbit	Gift from M. Smidt	1:500
TH	rabbit	Pel-Freeze	1:1000

Secondary antibodies:

Conjugant	Antigen	Species	Supplier	Concentration
Alexa Fluor 488	chicken IgG	goat	Molecular Probes	1:1000

Alexa Fluor 568	mouse IgG	goat	Molecular Probes	1:1000
Alexa Fluor 568	rabbit IgG	goat	Molecular Probes	1:1000

2.2.5. TUNEL labelling

Cryostat sections were processed as described for immunostaining with anti-GFP antibody, then treated according to manufacturers instructions using ApopTag Red In Situ Apoptosis Detection Kit (Chemicon).

2.2.6. Nissl staining

Wax sections from neonate brain were de-waxed in xylene (BDH) and hydrated to dH_2O by taking them through an alcohol series. Sections were stained in a 0.1% Cresyl fast violet (Sigma) solution for 5 minutes then rinsed in distilled water. The stain was differentiated by washing slides in 95% ethanol for 5-30 minutes and checking microscopically. Slides were then dehydrated and cleared in xylene before mounting in DPX mounting medium (Fisher Chemicals).

2.2.7. Quantitative analysis of immunolabelled cells

To determine the total number of TH and Pitx3-GFP immunolabelled cells present in the E12.5 and E14.5 midbrain, all labelled cells were counted in serial coronal sections (30 µm thick) of an entire midbrain. Total counts from four or five E12.5 and E14.5 embryos of each genotype (wild type, *Pitx3*-heterozygous (+/-) and *Pitx3*-homozygous (-/-) were averaged and standard deviations were calculated. At E12.5 cells do not exhibit typical SNc and VTA architecture and so were all counted together. At E14.5 many sections exhibit typical SNc and VTA architecture, however some sections crossing the rostral and caudal part of midbrain do not. This caused difficulties in terms of categorising labelled cells into either the SNc or VTA group accordingly for all sections. Therefore, to determine the relative number of DA neurons present in either SNc or VTA, we carried out comparative counts by examining 5 typical sections obtained for each genotype (Fig 2. C, D). Statistical analysis for the counting data was performed using two samples student *t*-test.

2.3. Molecular biology methods

2.3.1. Preparation of XL-1 blue competent cells by rubidium chloride method

Agar stock XL-1 blue cells were grown on LB agar plates with 20µg/ml tetracycline at 37°C overnight. Colonies were picked and each added to 2.5 ml LB media with 20µg/ml tetracycline and incubated in a shaker at 37°C overnight. The 2.5ml cultures were added to 250ml of LB media with 20mM $MgSO_4$ and incubated in a 1L flask in a shaker at 37°C for 5-6 hours, until the A_{600} reached 0.4 to 0.6. The 250ml cultures were centrifuged at 4500*g* for 5 minutes at 4ºC. From this stage the protocol was carried out in a room at 4°C with all equipment chilled. The cell pellet was gently resuspended in 0.4 volumes (100ml) of ice-cold solution TFB1 and incubated on ice for 5 minutes. The cells were centrifuged at 4500*g* for 5 minutes at 4°C. The cell pellet was gently resuspended in 1/25 original culture volume (10ml) of ice cold solution TFB2. The cells were incubated on ice for 15-60 minutes before aliquoting 200µl per tube and quick-freezing on dry ice. Tubes were then stored at -80°C.

2.3.2. Cloning

Cloning of DNA fragments was achieved via restriction enzyme digestion, gel purification and ligation. Restriction enzyme digestions were performed according to manufacturers instructions (all enzymes were from Roche or NEB). Digested DNA was separated by running on agarose gels (0.6 – 1.5% depending on size of fragment) in TAE buffer. The desired fragment was cut from the gel and the DNA extracted using Qiagen gel extraction kit (Qiagen) according to manufacturers instructions. The concentration of the recovered DNA was estimated by agarose electrophoresis. Ligations were performed with a 3:1 insert:vector molarity ratio with 1U of T4 DNA ligase (Roche) in 1X T4 ligation buffer (Roche) and incubated at 16°C overnight.

2.3.3. Transformation of competent cells

A 200µl aliquot of competent cells was thawed on ice. Approximately 10ng of plasmid DNA or ligation reaction was added in 1-3µl of dH_2O, gently stirred and incubated on ice for 30 minutes. The tube was heated at 42°C for 1 minute and then cooled on ice for 2 minutes. 1ml of LB was added to the tube and incubated in a shaker at 37°C for 1 hour. 50 to 300µl of the culture were plated on LB agar plates with appropriate selection and incubated at 37°C overnight.

2.3.4. Analysis of transformants

In order to check if a ligation has worked the following mini-prep protocol was used. Single colonies were used to inoculate a tube containing 3ml LB media with appropriate selection and incubated in a shaker at 37°C overnight. 1.5 ml of the

culture was centrifuged at 13000rpm for 3 minutes and the supernatant was discarded. 150µl of solution I was added to the tube and it was vortexed to mix well. To lyse the cells, 150µl of freshly prepared solution II was added and the tube was inverted 4-5 times to mix. To neutralise the lysate, 250µl of cold solution III was added and the tube was inverted 4-5 times to mix. The sample was then centrifuged at 13000 rpm for 15 minutes and the supernatant transferred to a clean tube. To precipitate the DNA, 1ml of cold ethanol was added and the tube was inverted 4-5 times to mix before incubating at -20°C for 30 minutes. The sample was then centrifuged at 13000rpm for 30 minutes to pellet the DNA, which was then dissolved in 50µl dH_2O to give a final concentration of about 2.5µg/ml.

2.3.5. Preparation of plasmid DNA

Qiagen mini-prep and midi-prep kits were used according to manufacturers instructions to prepare desired quantities of plasmid DNA.

2.3.6. Isolation of genomic DNA

Culture media was aspirated from ES cells and they were washed twice with PBS. Lysis buffer was added to the well (0.4ml per $2cm^2$ well) and plates were incubated at 37°C overnight. The following day the lysate was transferred to an eppendorf tube and an equal volume of isopropanol was added. The tube was gently inverted a few times until the DNA was visible then the sample was centrifuged in a bench top centrifuge at 14000 rpm for 20 minutes at 4°C.

2.3.7. Southern hybridisation

20µg of genomic DNA were digested with 200 units of restriction enzyme in the appropriate buffer and temperature overnight. Digested DNA was precipitated by adding 3 volumes of ethanol (BDH) and 1/10 volume 3M Na Acetate and leaving at -20°C overnight. Samples were centrifuged at 14000rpm at 4°C for 30 mins then washed with 70% ethanol, before dissolving DNA in 20µl dH_2O. The digested DNA was run on a 0.8% agarose gel in TAE buffer overnight. The gel was placed in depurination solution for 10 minutes (or until the bromophenol blue turned yellow) with gentle agitation then rinsed with dH_2O. The DNA was denatured to allow its transfer to the membrane by placing the gel in denaturation solution for 30 minutes with gentle agitation. The gel was then blotted by capillary transfer onto a positively charged nylon membrane (Hybond N+, Amersham) overnight in denaturation solution. Following transfer the membrane was baked in an oven at 80°C for 2 hours. Then membrane was either stored in an air tight container at room temperature or

used immediately for hybridisation. For hybridisation, the membrane was rinsed in 2X SSC and placed in a hybridisation tube with 30ml prehybridisation solution for 2 hours to prevent non-specific binding. The radiolabelled probe was prepared by denaturing a solution containing 30ng of plasmid DNA fragment in 11µl dH_2O by boiling for 10 minutes and then chilling on ice for 5 minutes. Then added to the tube was 4µl of High Prime (Roche) and 5µl α-^{32}P-labelled dCTP (Amersham), and it was incubated at 37°C for 30 minutes before adding 2µl 0.2M EDTA and 28 µl STE buffer to stop the reaction. The sample was then put in a ProbeQuant G-50 Microcolumn (Amersham) and centrifuged at 300rpm for 2 minutes and the eluate containing the probe was reserved. Before adding the probe to the hybridisation solution it was denatured at 100°C for 10 minutes. The membrane was then incubated with 15 ml hybridisation solution containing the denatured probe in a rotating tube at 65°C overnight. The membrane was washed with 2X to 0.5X SSC with 0.1% SDS to remove non-bound radiolabelled probe and then the labelled target sequence was visualised using autoradiography (Kodak film).

2.4. Cell culture

Generally cells were grown in tissue culture plastics (Iwaki) coated with 0.1% gelatine (Sigma) and incubated in at 37°C with 7% CO2 in a humidified incubator (Sanyo). All solutions were sterility tested before use. To avoid bacterial and fungal contamination, all tissue culture manipulations were undertaken inside a laminar flow sterile hood (Microflow) and surfaces and objects were sprayed with 70% industrial methylated spirits (IMS) before use.

2.4.1. Primary culture

E14.5 midbrains were dissected, then were manually dissociated by trituration in GMEM media supplemented 100 U/ml penicillin (Invitrogen) and 100 µg/ml streptomycin (Invitrogen). Cells were plated at a density of $4x10^5$ cells/ml and cultured on a layer of PA6 stromal cells (as described below) for 48 hours, then were fixed and processed for immunohistochemistry.

2.4.2. PA6 stromal cell culture

PA6 cells were cultured on 0.1% gelatine-coated tissue culture flasks in GMEM.

2.4.3. Routine culture of ES cells

ES cells were cultured on 0.1% gelatine-coated tissue culture flasks or plates in Glasgow modified Eagle's medium (GMEM) supplemented with 100 units/ml leukaemia inhibitory factor (LIF). LIF was prepared in house by transfecting COS-7 cells with a human LIF expression plasmid and harvesting the medium. The concentration of LIF was assayed using CP1 indicator cells (LIF preparation was performed by L. Taylor). All media and solutions were tested for contamination 3 days before use. When passaging and thawing cells, all media and solutions were prewarmed to 37°C.

2.4.3.1. Passaging ES cells

To passage the cells the media was removed using an aspirator then cells were washed twice with PBS. 1X (0.025%) trypsin (Invitrogen) was added to just cover the cell monolayer and the flask was incubated at 37°C for about 1 minute. The trypsin activity was neutralised by addition of media, 4mls media was added for every 1ml of 1X trypsin. The cell suspension was transferred to a universal tube and centrifuged for 5 minutes at 1200rpm. Cells were then resuspended in media to achieve a single cell suspension, counted using a haemocytometer and replated at a density of 10^6 cells per 25cm^2 flask. Routinely cells were passaged every 2-3 days.

2.4.3.2. Freezing ES cells

Cells in tissue culture flasks were trypsinised as before and centrifuged for 5 minutes at 1000rpm. Cells were resuspended in 1ml of freezing mix (10% dimethyl sulphoxide (DMSO) in normal culture media) per 25cm^2 flask and transferred into a cryotube (Nunc) at 0.5ml per vial. Cryotubes were placed at -80°C overnight and then transferred to a liquid nitrogen cell bank (Series 2300, Custom biogenic Systems).

To freeze cells in 24-well tissue culture plates (they should be 70% confluent), the media was removed and replaced with just enough freezing mix to cover the layer. The plate was then placed in a polystyrene box and stored at -80°C.

2.4.3.3. Thawing ES cells

Frozen cryotubes were retrieved from liquid nitrogen storage and placed immediately into 37°C waterbath. Once the cells were thawed they were transferred into 9.5 ml warm media to dilute out the DMSO. The cell suspension was then centrifuged for 5 minutes at 1200rpm and then the cells were gently resuspended in 10mls of media

and transferred to a 25cm^2 flask. The media was then refreshed about 10 hours later to remove any dead cells and dilute out the remaining DMSO.

To thaw frozen 24-well plates, pre-warmed media was added to fill the wells and the bottom of the plate was warmed by hand. Once thawed the media was carefully removed, leaving just enough to cover the layer of cells and 0.5ml fresh media was added.

2.4.5. Diploid aggregation method

The method used was based on that of Nagy and Rossant (Nagy and Rossant, 1993). Briefly, eight-cell stage embryos were flushed from the oviducts of 2.5 days post-coitum (dpc) superovulated F1 females (PMS <48hrs 0.0 dpc, HCG 0.0 dpc). The zona pellucida was removed and they were incubated with small clumps of ES cells (8-15 ES cells per embryo) overnight. Embryos were then transferred to 2.5 dpc pseudopregnant F1 recipients, a maximum of 8-10 embryos were transplanted into each uterine horn.

2.5. Gene targeting

2.5.1. Transfection and selection of recombinant ES cells

Three 150cm^2 confluent flasks of E14TG2a ES cells at passage 12 were harvested by trypsinisation and pooled to give a total of 6x10^7 cells. At this stage 2x10^6 cells were plated on two 10cm plates as controls. The remaining cells were centrifuged for 5 minutes at 1200rpm and resuspended in 700µl of PBS. The cells were transferred to a sterile electroporation cuvette and 50µg of linearised targeting vector DNA in 100µl PBS was added. Cells were then electroporated at 800V and 3µF for 0.1 seconds (BioRad Gene Pulser). Immediately after electroporation the cells were resuspended in media and plated on 18 10cm^2 plates at a density of 2x10^6 cells per plate. 24 hours later HygromycinB was added to the plates at a concentration of 100µg/ml (9 plates) and 150µg/ml (9 plates). Media with HygromycinB selection was refreshed every other day. When appropriate (about 10 days), single HygromycinB

resistant colonies were picked into 24-well plates and expanded. Each clone was split into two 24-well plates, one of which was frozen and the other was used for genomic DNA extraction for Southern blotting.

2.5.2. Transient Cre expression in ES cells

A recombinant clone was expanded to a 25 cm^2 flask and trypsinised as before. Cells were centrifuged for 5 minutes at 1200rpm and resuspended in 700µl of PBS. Transient Cre expression was achieved using a plasmid (pCAGCre) containing Cre under the control of the cytomegalovirus/chicken beta-actin (CAG) promoter, which produces efficient recombination by Cre (Araki et al., 1997). The cells were transferred to a sterile electroporation cuvette and 10µg of Cre plasmid (pCAGCre) in 100µl of PBS was added. Cells were then electroporated at 800V and 3µF for 0.1 second (BioRad Gene Pulser). Immediately after electroporation the cells were resuspended in media and plated on six 10cm^2 plates at a densities of $1x10^4$ cells per plate (two plates), $3x10^3$ cells per plate (two plates) and $1x10^3$ cells per plate (two plates). After 48 hours, ganciclovir selection was added to the plates at a concentration of 2.5µM. Media with ganciclovir selection was refreshed every other day. When appropriate (about 10 days), single ganciclovir resistant colonies were picked into two 24-well plates (one to freeze and one to extract DNA for Southern blot) and expanded.

Chapter 3

Pitx3-GFP mirrors *Pitx3* RNA and protein expression

3.1. Introduction

In order to visualise and follow the expression of Pitx3, a reporter protein was linked to the endogenous expression of Pitx3. Reporter proteins can generally be visualised due to bioluminescence, fluorescence or enzymatic properties. The gene encoding the reporter protein (the reporter gene) may be expressed under the control of a specific promoter by a number of methods, for example, in a traditional transgenic approach a construct can be made containing a cell type-specific promoter fused to a reporter gene. The DNA construct containing the promoter and reporter gene is directly injected into one of the pronuclei of a single celled zygote and the DNA integrates stably into the genome. However, this integration is random and neighbouring sequences may interfere with the regulation of the reporter gene expression. These position effects can obscure analysis of reporter gene expression, therefore, this approach is not the most reliable for analysing gene expression. To avoid this problem, the reporter gene may be placed under the control of a promoter of an endogenous gene via homologous recombination in ES cells. The main advantage of this knock-in approach is that the reporter gene is under the control of the complete set of endogenous *cis*–acting regulatory elements that control the endogenous gene expression.

A number of proteins have been successfully used as reporter proteins, these include the enzymes β-galactosidase (β-gal) and luciferase. Enzymatic reporters are sensitive detectors of gene expression as each molecule of enzyme can catalyse many reactions to produce the detected product. However, a substrate must be present in order to facilitate the visualisation of the reporter expression and it can be difficult to deliver the substrate to live cells. Another option is the use of a vital fluorescent reporter such as green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. Several mutants of wild-type GFP have been created to allow increased brightness, thermostability, optimised codon usage for mammalian expression and different spectral properties. For example, the most commonly used variant eGFP has two amino acid changes in the vicinity of the fluorophore (Phe-64 to Leu and Ser-65 to Thr), thus it is codon optimised and is 35-fold brighter than wild-

type GFP (Hadjantonakis et al., 2003). Whilst eGFP may not be as sensitive as some enzymatic reporters it has many advantages including visualisation in both live and fixed tissues and cells, no substrate or reaction cofactors are required and commercial antibodies are available. Thus, eGFP was the reporter protein of choice in the making of *Pitx3* reporter mice.

Prior to the start of this PhD thesis, mice were created with an enhanced GFP (eGFP) reporter targeted into the *Pitx3* locus via homologous recombination in ES cells. The entire *Pitx3* coding sequence (exons 2, 3 and 4) was replaced with an eGFP*irespac* cassette downstream of the endogenous *Pitx3* promoter (Fig. 3.1) (Zhao et al., 2004). Therefore, eGFP expression is under the control of endogenous *Pitx3* regulatory elements and there is no expression of Pitx3 from the mutated allele. Mice that are heterozygous and homozygous for this *Pitx3*-GFP allele have been generated from these ES cells and they are both viable, able to survive to adulthood and fertile. In addition, *Pitx3*-heterozygous mice have no apparent phenotypic abnormality (see Ch. 5). The first aim of this study was to investigate whether the expression of the *Pitx3*-GFP reporter faithfully mirrors that of reported Pitx3 expression in mouse embryos and in the adult mDA system.

3.2. Results

3.2.1. Direct visualisation of *Pitx3*-GFP in heterozygous embryos

Previous studies reported that Pitx3 mRNA was expressed in the eye lens, midbrain, tongue, incisor primordia, mesenchyme around the sternum and vertebrae and in the head muscles (Semina et al., 1998; Smidt et al., 1997). To investigate whether the expression of the *Pitx3*-GFP reporter replicated Pitx3 expression, GFP was visualised in phenotypically normal *Pitx3*-heterozygous embryos. Embryos between the ages E10.5 and E14.5 were either taken immediately for whole mount visualisation or fixed and sectioned at 100µm on a vibratome (Ch. 2.2). In the brain, *Pitx3*-GFP expression was confined to the ventral midbrain and was first visible at E12.5 as a small domain on the ventral mesencephalic flexure (Fig. 3.2. A). From E12.5 to E13.5 there is a significant increase in the *Pitx3*-GFP expressing domain in the ventral midbrain (Fig. 3.2. B). At E14.5 *Pitx3*-GFP expression can be observed in the cell bodies of ventral midbrain neurons and also in their axons projecting to their target sites in the striatum and

basal forebrain (Fig. 3.2. C). *Pitx3*-GFP expression was also present in the lens, where it could be first detected at E10.5 in the lens primordium (Fig. 3.3. A) and expression remained in the lens throughout life (Fig. 3.3. B). In addition, *Pitx3*-GFP expression was visible in the somites (Fig. 3.3. C), cranial facial muscles and the tongue (Fig. 3.3. D).

3.2.2. *Pitx3*-GFP mirrors Pitx3 protein expression

Direct visualisation of the GFP reporter described above established that *Pitx3*-GFP was expressed in the area where mDA neurons arise and where Pitx3 expression had been reported previously. To directly demonstrate that *Pitx3*-GFP expression reports that of Pitx3 protein expression in mDA neurons, Pitx3 antibody staining was performed along with antibody staining for GFP on *Pitx3*-heterozygous midbrains at E12.5. In all *Pitx3* hetrozygous midbrain sections studied, the *Pitx3*-GFP expressing cells were also positive for Pitx3 antibody and vice-versa (Fig. 3.4). This confirms that the *Pitx3*-GFP reporter accurately replicates Pitx3 protein expression in mDA neurons. Therefore, *Pitx3*-GFP could be used as a lineage marker to track the fate of Pitx3-expressing cells in both the *Pitx3* heterozygous and *Pitx3* null mice.

3.2.3. Pitx3 is expressed exclusively in mDA neurons of adult mice

Continued expression of Pitx3 in mDA neurons in adulthood has been reported before and during the course of this study (Hwang et al., 2003; Smidt et al., 2004; Smidt et al., 1997; Van Den Munckhof et al., 2003). To validate the expression of the *Pitx3*-GFP reporter in adult mDA neurons, sections of adult midbrain were stained with antibodies against GFP and TH (Fig. 3.5). Counting of labelled cells in eight randomly chosen fields in the SNc and VTA of three adult brain samples revealed that the vast majority of TH-expressing cells express *Pitx3*-GFP and vice-versa (Table 3.1). This was the case in both the SNc and in the VTA. In the SNc 98.53 ± 2.35 of TH^+ cells expressed *Pitx3*-GFP and 99.36 ±0.9% of *Pitx3*-GFP^+ cells expressed TH. Similarly 94.6 ± 8.5% of TH^+ cells in the VTA expressed *Pitx3*-GFP and 94.9 ± 6.4% of *Pitx3*-GFP^+ cells expressed TH. The few cells that did not appear to co-express Pitx3 and TH were not localised in any region and were scattered throughout the SNc and VTA. A possible reason for the detection of some Pitx3- or TH-only expressing cells may be due to the plane of the section, as the TH is localised to the cytoplasm whereas GFP is present in the nucleus and the cytoplasm.

	Number of cells counted	% of TH expressing *Pitx3*-GFP	% of *Pitx3*-GFP expressing TH
SNc	884	98.53 ± 2.3	99.36 ±0.9
VTA	457	94.6 ± 8.5	94.9 ± 6.4

Table 3.1. Quantitative analysis of co-expression of *Pitx3*-GFP and TH in adult mDA neurons

Data was collected from eight randomly chosen fields from three adult brain samples. Values are mean ± standard deviation.

3.3. Summary

The examination of embryos and adult mice heterozygous for the *Pitx3*-GFP allele revealed that *Pitx3*-GFP was an accurate reporter of Pitx3 expression. In embryos *Pitx3*-GFP expression was observed in midbrain, eye, somites, cranial facial muscles and the tongue, which correlates with reported sites of *Pitx3* mRNA expression (Semina et al., 2000; Semina et al., 1997; Smidt et al., 1997). Immunostaining with an antibody against Pitx3 was performed in conjunction with an antibody against GFP which revealed that *Pitx3*-GFP accurately mirrors Pitx3 protein expression in E12.5 *Pitx3* heterozygous midbrain. Furthermore, in adult mice *Pitx3*-GFP was found to be co-expressed with TH in virtually all mDA cells of the SNc and VTA.

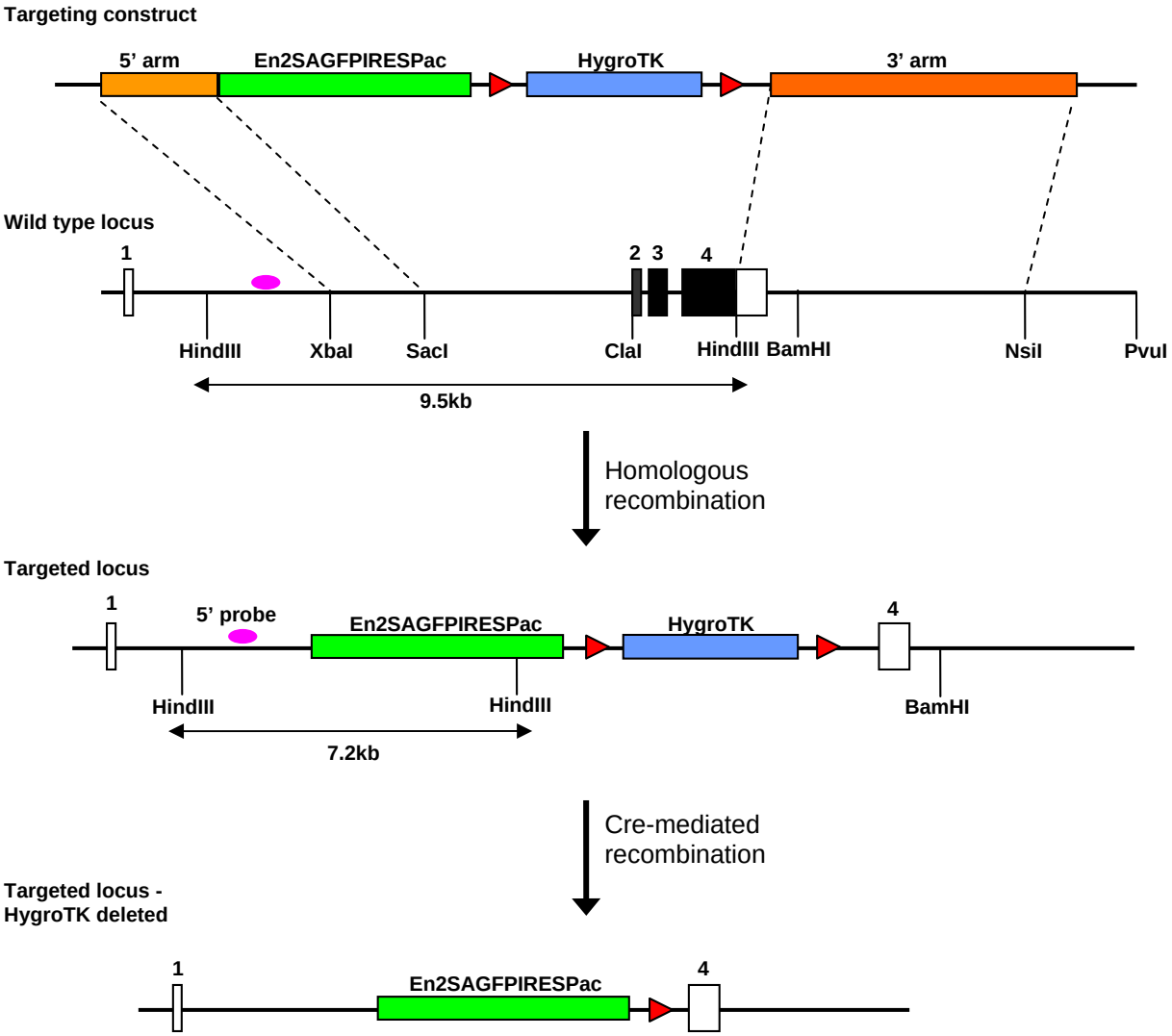

Figure 3.1. Targeting of the *Pitx3* locus to produce *Pitx3*-GFP.

Shown at the top is the Pitx3 targeting vector which contains an engrailed 2 splice acceptor (SA), an eGFP reporter, an internal ribosomal entry site (IRES) and a puromycin resistance gene (pac)-polyA cassette. In addition, a floxed phosphoglycerate kinase (PGK) promoter-driven hygromycin-thymidine kinase (HygroTK) cassette is included to allow for selection of ES cell transfectants and is later removed via Cre-mediated recombination. The 5' homologous arm is 1.8kb and the 3' homologous arm is 6kb. The homologous areas are indicated and so is the targeted locus following a replacement homologous recombination event. Cre-mediated excision of the PGK-HygroTK cassette results in the final targeted locus. The four boxes represent Pitx3 exons and the filled regions indicate coding sequence, whilst the blank regions represent non-coding sequence.

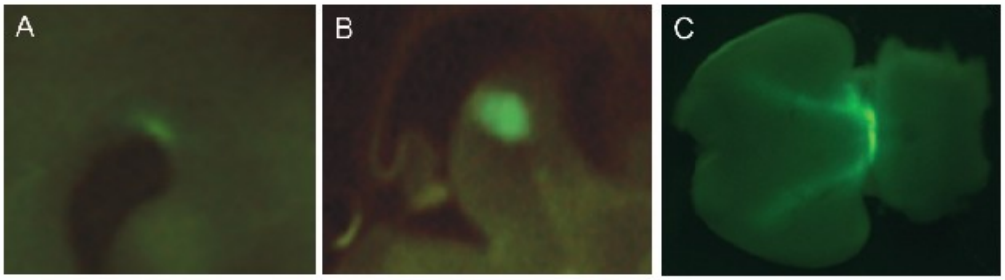

Figure 3.2. Direct visualisation of *Pitx3*-GFP in the CNS of *Pitx3* heterozygous embryos.

Whole mount or 100µm vibrotome sectioned embryos were used to reveal the expression of *Pitx3*-GFP. *Pitx3*-GFP expression is first seen in the midbrain at E12.5 and appears as a small group of cells on the ventral surface of the mesencephalic flexure (A). By E13.5 this group of *Pitx3*-GFP expressing cells in the midbrain is larger (B). A ventral view of a whole E14.5 midbrain shows the cluster of *Pitx3*-GFP cells in the ventral midbrain and projections towards their forebrain targets (C).

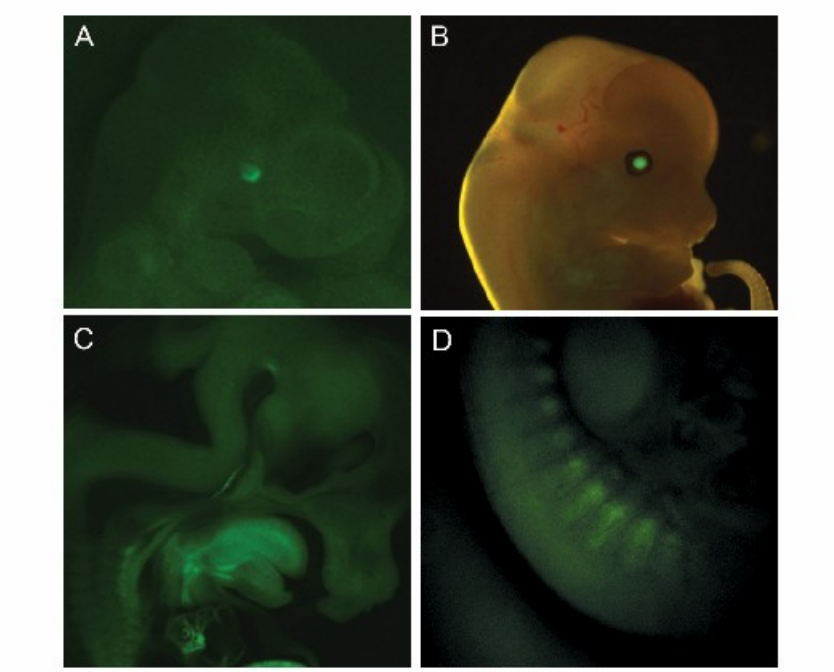

Figure 3.3. Direct visualisation of *Pitx3*-GFP in non-CNS areas of *Pitx3* heterozygous embryos.

A significant non-CNS site of Pitx3 expression is the lens where *Pitx3*-GFP expression is visible in the lens primordium at E10.5 (A) and in the lens at E12.5 (B). Additionally *Pitx3*-GFP expression was detected in the somites at E12.5 (C) and in the tongue and head mesenchyme at E12.5 (D).

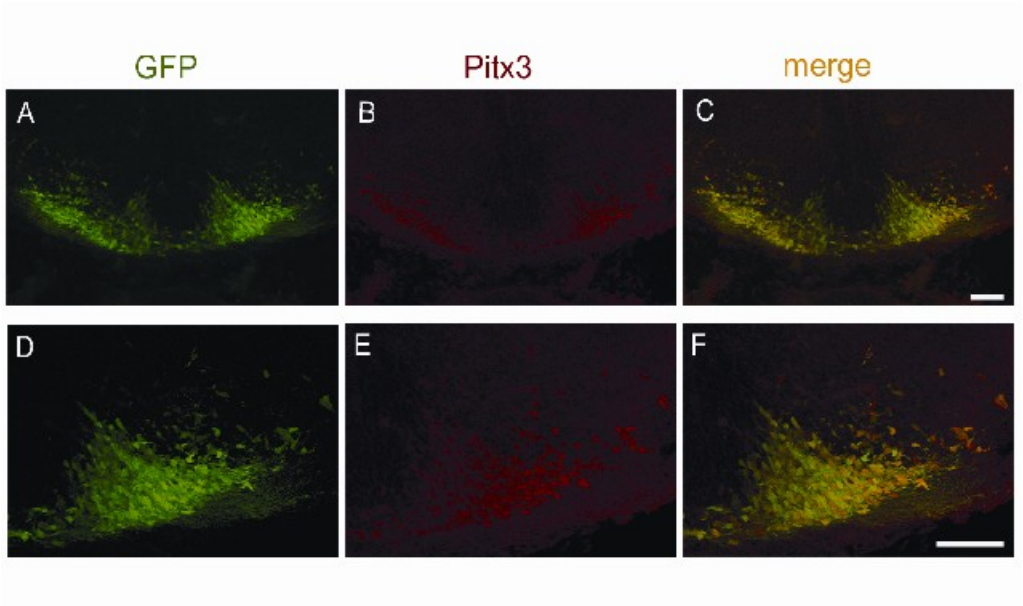

Figure 3.4. Immunostaining for Pitx3 and *Pitx3*-GFP on E12.5 midbrain sections.

Antibodies against Pitx3 and GFP were used on 30µm E12.5 midbrain sections. All *Pitx3*-GFP positive cells (green) are also positive for Pitx3 antibody (red) and vice-versa. Panel shows x20 magnification (A-C) and x40 magnification (D-F). Scale bars 200µm.

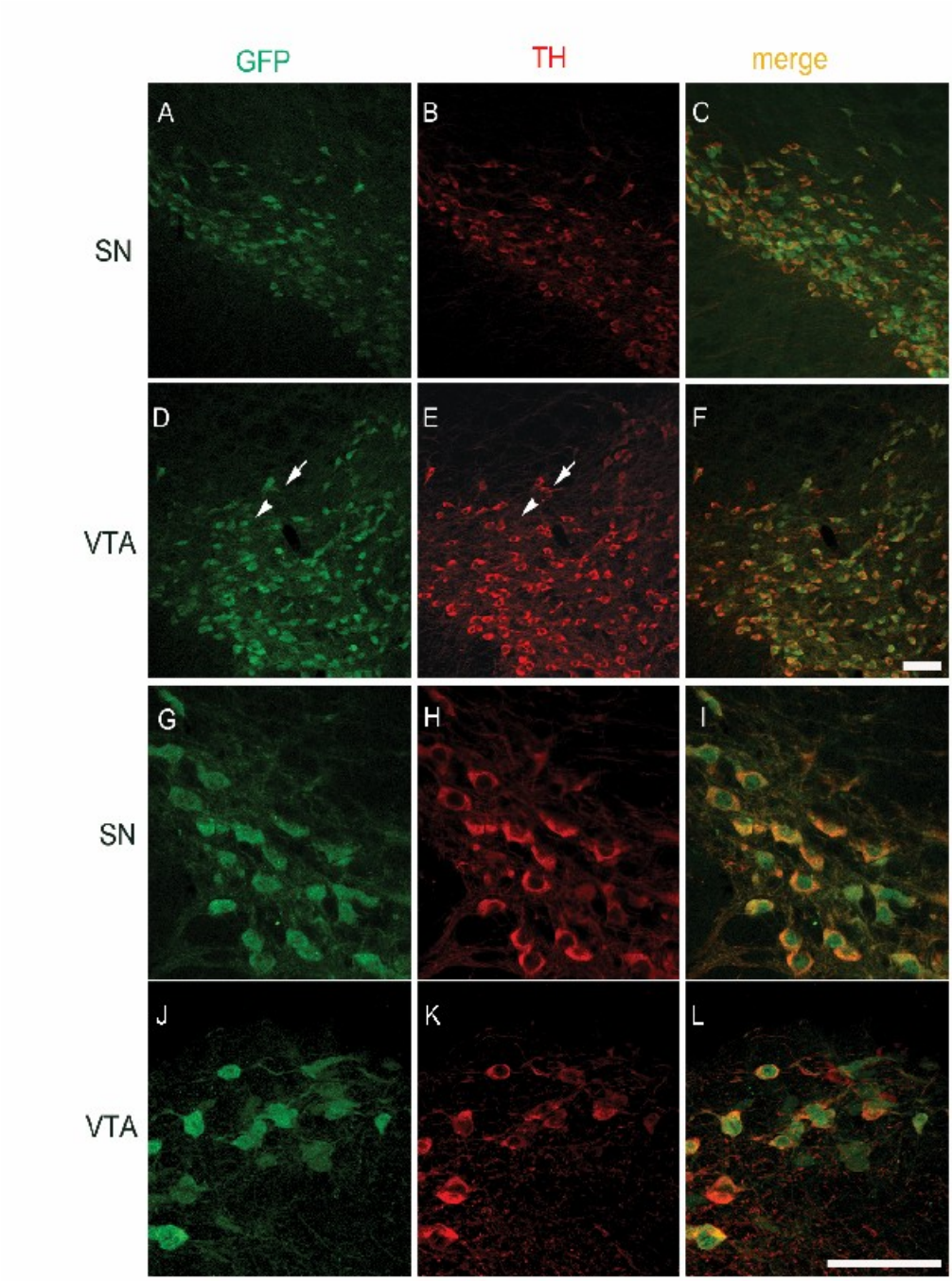

Figure 3.5. Co-expression of *Pitx3*-GFP and TH on adult midbrain sections. Examples of mDA neurons in SNc (A-C, G-I) and VTA (D-F, J-L) from adult midbrain 30µm coronal sections stained with antibodies against GFP and TH. In both the SNc and VTA approximately all *Pitx3*-GFP^{+} cells (green) also express TH (red) and vice-versa. There was the occasional cell that did not co-express Pitx3 and TH, examples of these are indicated by arrows pointing to Pitx3-GFP^{+} only cells (D) and TH^{+} only cells (E). Scale bars 100µm.

Chapter 4

Identification of ontogenetically distinct subgroups of mDA neurons

4.1. Introduction

The reported expression of *Pitx3* mRNA in the midbrain begins at E11.5 (Smidt et al., 1997), Pitx3 protein can be detected from E12.5 (Ch. 3) in mDA neurons and the espression of Pitx3 in mDA neurons continues into adulthood. The expression of TH in mDA neurons begins at around E11.5 (Foster et al., 1988; Solberg et al., 1993; Zetterstrom et al., 1997) and the presence of this key enzyme in dopamine synthesis is indicative of a dopaminergic phenotype. Between researchers there are differing definitions of the features that identify a dopaminergic neuron and a dopaminergic neuron progenitor. However, throughout this thesis a dopaminergic neuron will be defined as one that expresses TH. In addition, a post-mitotic neuron that is destined to become a mDA neuron but has not yet started to express TH will be described as a post-mitotic mDA neuron progenitor. Until now studies relating to the co-expression of Pitx3 and TH in early mDA neuron development had not been conducted. Therefore, it was not clear whether the earliest Pitx3-expressing cells were dopaminergic neurons (i.e. TH-positive) or non-dopaminergic (i.e. TH-negative) cells that will become mDA neurons. To address this issue, the pattern of Pitx3 and TH expression in the ventral midbrain from E12.5 to E14.5 was examined. These stages were chosen because the majority of mDA neurons emerge between E12.5 and E14.5 (Altman and Bayer, 1981; Hanaway et al., 1971; Lauder and Bloom, 1974) and the first stage at which *Pitx3*-GFP expression can be detected is E12.5 (Ch. 3.2).

4.2. Results

4.2.1. Analysis of Pitx3-GFP and TH expression in the developing midbrain at E12.5, E13.5 and E14.5

Serial midbrain sections spanning the Pitx3 and TH expression domain from *Pitx3*-heterozygous mice were stained with antibodies against GFP and TH. The Pitx3-GFP reporter could be exploited in this situation to detect Pitx3 expression as the *Pitx3* heterozygous mice were shown to be phenotypically normal (Ch. 5.2). The earliest stage examined was E12.5 as this was the first stage at which *Pitx3*-GFP expression could be detected in the midbrain. This revealed that *Pitx3*-GFP and TH were differentially expressed in some midbrain cells and co-expressed in other midbrain cells at E12.5. Some cells express TH but not Pitx3, whilst another population of cells express Pitx3 but not TH and some cells express both TH and Pitx3. At the most rostral level of the E12.5 midbrain, the *Pitx3*-GFP and TH expression domains are in a relatively lateral position and at the most caudal level however, these domains are positioned relatively medially (Fig. 4.1). Rostrally, the $Pitx3^{+}TH^{-}$ population of cells is in a more lateral position relative to the $Pitx3^{-}TH^{+}$ expressing population (Fig. 4.1 A-B). Moving caudally, the *Pitx3*-GFP and TH expression domains remain partially overlapped and the $Pitx3^{+}TH^{-}$ population is more ventral and lateral relative to the $Pitx3^{-}TH^{+}$ population (Fig. 4.1 C-F). Further caudally, the *Pitx3*-GFP and TH expression domains overlap more so there are more Pitx3+TH+ cells (Fig. 4.1 G-H). At this caudal position the *Pitx3*-GFP and TH expression domains resemble the VTA.

At E13.5, a large proportion of cells are positive for both Pitx3 and TH expression and there are more of both *Pitx3*-GFP^{+} and TH^{+} cells compared to E12.5 (Fig.4.2). This suggests that many of the cells that expressed only *Pitx3*-GFP or TH at E12.5 have now acquired TH or *Pitx3*-GFP expression, respectively. By E14.5, there are greater numbers of *Pitx3*-$GFP^{+}TH^{+}$ cells as compared to E13.5 and almost all mDA cells express both *Pitx3*-GFP and TH (Fig. 4.3) with the exception of some cells in the rostral lateral primordium, which still express only *Pitx3*-GFP (Fig. 4.3). By E14.5 the collection of mDA neurons resembles that of the adult SNc and VTA and the few $Pitx3^{+}$ cells that do not express TH are located in lateral most part of the primitive SNc. Eventually, by adulthood virtually all mDA neurons of the SNc and VTA express both *Pitx3*-GFP and TH (Ch. 3.3).

4.2.2. Characterisation of *Pitx3*-GFP cells at E12.5

At E12.5 many of the *Pitx3*-GFP expressing cells do not express TH. To investigate whether these cells are likely to be mDA progenitors, further immunohistochemical analysis was carried out on these cells at E12.5. Markers for mitotic progenitors (Ki67, labels cells in G1, G2 and M phase), nascent neurons (βTubulin3), midbrain dopaminergic neurons and their progenitors (En1), GABAergic neurons (GAD) and glial cells (S100β and GFAP) were examined. Antibody staining for the neuronal marker βTubulin3 revealed that the majority of *Pitx3*-GFP cells were neurons (Fig. 4.4). Some medially positioned *Pitx3*-GFP cells did not express βTubulin3, which may be due to their recent exit from the cell cycle and consequently they have not yet begun to express this neuronal marker. None of the *Pitx3*-GFP expressing cells expressed the proliferative marker Ki67 (Fig. 4.4), suggesting that all *Pitx3*-GFP cells are post-mitotic. As the main wave of neurogenesis in mouse occurs from around E13 and is followed by gliogenesis from around E17, it is not expected that the *Pitx3*-GFP cells at E12.5 will express glial cell markers. The expression of the early glial cell marker S100β begins at E13.5 in mouse brain restricted to the medulla and spinal cord (Vives et al., 2003) and glial fibrillary acidic protein (GFAP) protein is first detected in the mouse brain at E17 (Andrae et al., 2001). These markers, S100β and GFAP, were not observed in any *Pitx3*-GFP expressing cells at E12.5 (data not shown), suggesting that all *Pitx3*-GFP cells are neurons or neuron progenitors. Key evidence that the *Pitx3*-GFP cells actually are mDA progenitors is that the majority of *Pitx3*-GFP expressing cells also expressed En1 (Fig. 4.4), which is an early mDA marker expressed in all mDA neurons from early development through into adulthood (Simon et al., 2001). Other midbrain neuronal subtype markers for example glutamic acid decarboxylase (GAD) a marker for GABAergic neurons was not found to be expressed in *Pitx3*-GFP cells (Fig. 4.4). Taken together, these data suggest that at E12.5 the *Pitx3*-GFP positive cells are post-mitotic mDA neuron progenitors.

4.2.3. Migration pattern of mDA neurons

At E12.5 it is difficult to assess which cells will form the SNc and which will form the VTA as the structure formed by the $Pitx3^+$ and TH^+ cells in the midbrain does not accurately represent the adult SNc and VTA. Therefore, in order to understand more about the situation and likely fate of the midbrain cells at E12.5 it is useful to understand their migration pattern. Presently the data available on migration patterns of these neurons is ambiguous and there are different opinions on the true mode of migration (see Ch. 8.5). To estimate the likely migration pattern of these early mDA

neurons, their orientation at E12.5 was examined. The bipolar orientation of the TH^+ cells at E12.5 revealed that cells located closer to the medial part of the neuroepithelium exhibited a vertical orientation (Fig 4.5.B), suggestive of a vertical migration route. Whereas cells located in a lateral position had a more horizontal orientation (Fig. 4.5.C), suggestive of a horizontal migration route. This data points to a pattern of migration which involves the cells migrating down the midline first ventrally from the neuroepithelium, then horizontally towards the lateral areas of the mDA region.

4.5. Summary

Studies on TH and Pitx3 expression throughout the midbrain in early stages of mDA neuron generation have revealed that at E12.5 there are distinct subpopulations of mDA neurons with different TH and Pitx3 expression profiles. Some mDA neurons in the midbrain at E12.5 express TH but not Pitx3, whilst another population of cells express Pitx3 but not TH and these two populations of cells partially overlap so that some cells express both TH and Pitx3. The $Pitx3^+TH^-$ cells are generally located in a relatively more lateral and ventral position, which suggests that they represent a developing SNc. However, as development progresses more midbrain cells express both TH and Pitx3 and by adulthood virtually all mDA cells that express TH also express Pitx3 and vice versa. The midbrain cells that express Pitx3 at E12.5 are likely to be post-mitotic mDA progenitors as they express markers for nascent neurons (βTubulin3) and midbrain dopaminergic neurons and their progenitors (En1). In addition, these Pitx3-expressing cells do not express markers for mitotic progenitors (Ki67), glial cells (S100β and GFAP) or GABAergic neurons (GAD). The bipolar orientation of the mDA cells at E12.5 is suggestive of a migration pattern vertically downwards from the midline of the neuroepithelium before lateral migration to their final position in the midbrain.

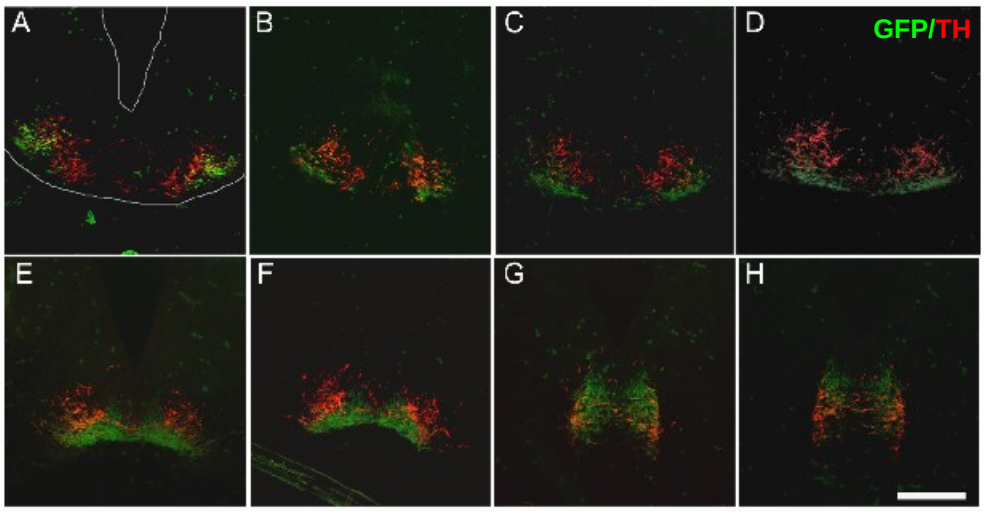

Figure 4.1. Series of sections through an E12.5 *Pitx3* heterozygous midbrain. Rostral to caudal coronal E12.5 midbrain sections stained with antibodies against GFP and TH (A-H). In the rostral sections the *Pitx3*-GFP and TH expression domains are in a relatively lateral position, however the $Pitx3^{+}TH^{-}$ population of cells is in a more lateral position relative to the $Pitx3^{-}TH^{+}$ expressing population (A-B). More caudally, the $Pitx3^{+}TH^{-}$ population is more ventral and lateral relative to the $Pitx3^{-}TH^{+}$ population (C-F). Further caudally, the *Pitx3*-GFP and TH expression domains are positioned relatively medially and many of the cells are $Pitx3^{+}TH^{+}$ (G-H). Scale bar is 200µm.

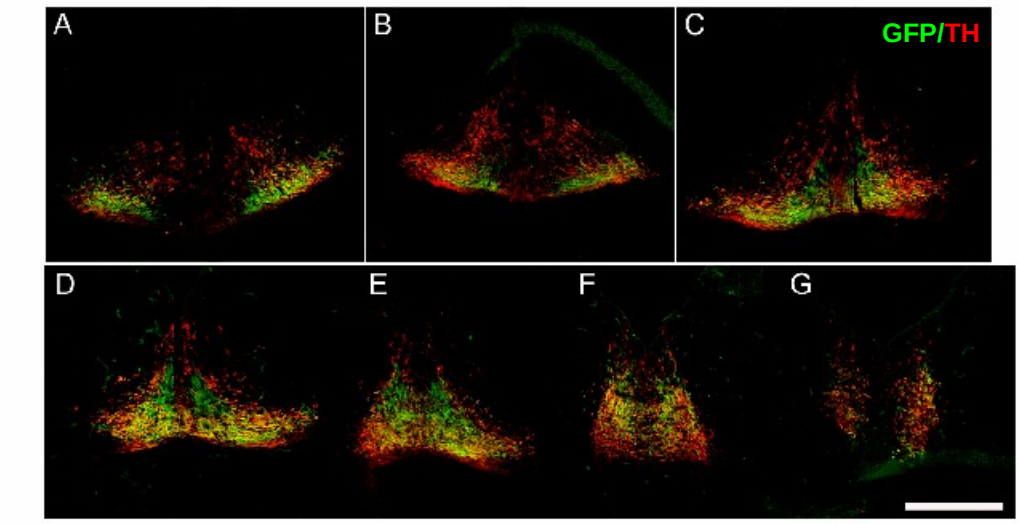

Figure 4.2. Series of sections through an E13.5 *Pitx3* heterozygous midbrain. Rostral to caudal coronal E13.5 midbrain sections stained with antibodies against GFP and TH (A-G). Many of the cells in these sections are $Pitx3^{+}TH^{+}$. In the rostral sections the cells are located in a relatively lateral position with some $Pitx3^{+}TH^{-}$ cells in a ventral position relative to the $Pitx3^{-}TH^{+}$ cells (A-C). More caudally the majority of the cells are $Pitx3^{+}TH^{+}$ and located in relatively medial position (D-G). Scale bar is 200µm.

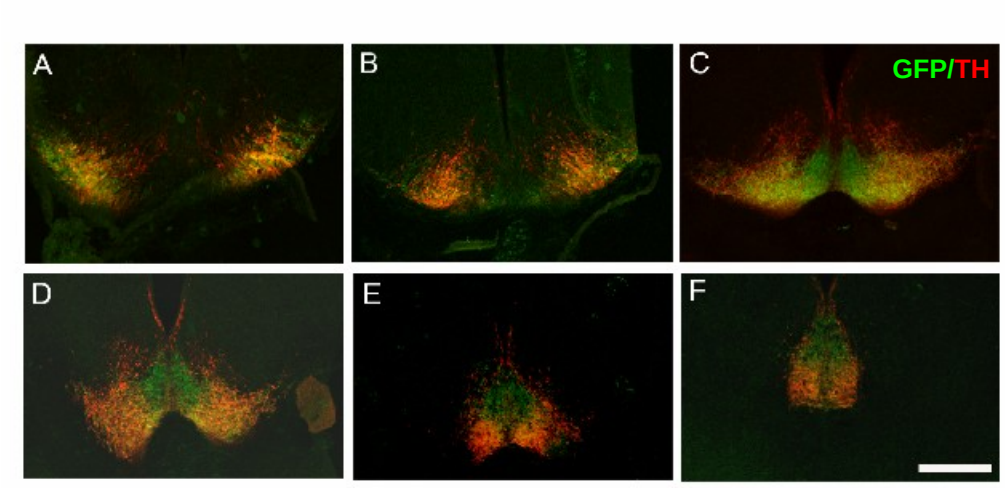

Figure 4.3. Series of sections through an E14.5 *Pitx3* heterozygous midbrain. Rostral to caudal coronal E14.5 midbrain sections stained with antibodies against GFP and TH (A-F). The majority of the cells are $Pitx3^{+}TH^{+}$ at this stage, however some $Pitx3^{+}TH^{-}$ cells remain in a relatively lateral position in the rostral most sections (A-B). Scale bar is 200µm.

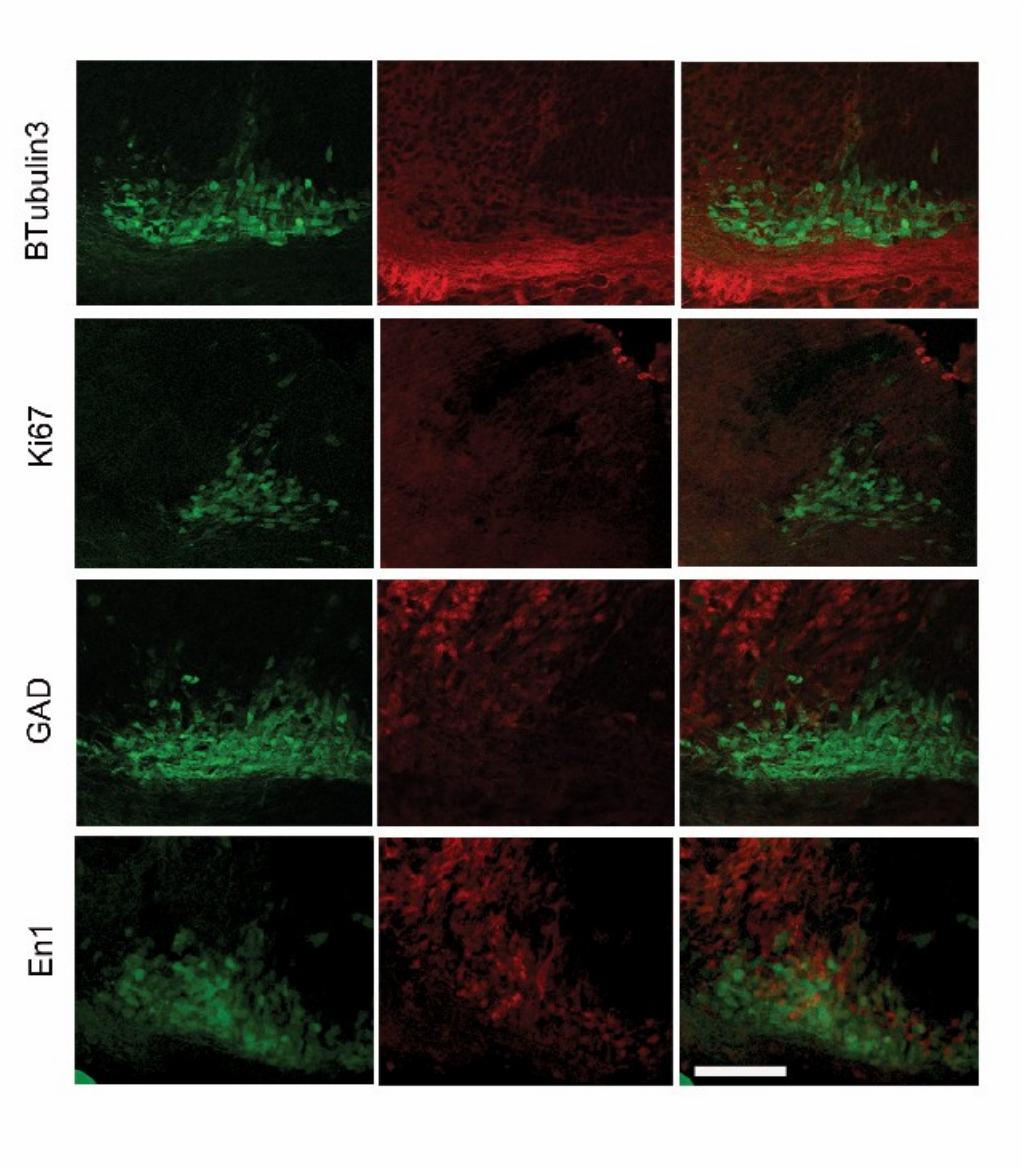

Figure 4.4. Immunohistochemical characterisation of E12.5 *Pitx3*-GFP cells.
Coronal midbrain sections from E12.5 *Pitx3* heterozygous embryos were immunostained with anti-GFP (green, left panel) combined with either anti-ßTubulin3, Ki67, GAD or En1 (red, middle panel) antibodies, merged image is shown in the right panel. All images show the left side of the section, in which the ventricular zone is at the top right of the image. Scale bar is 100µm.

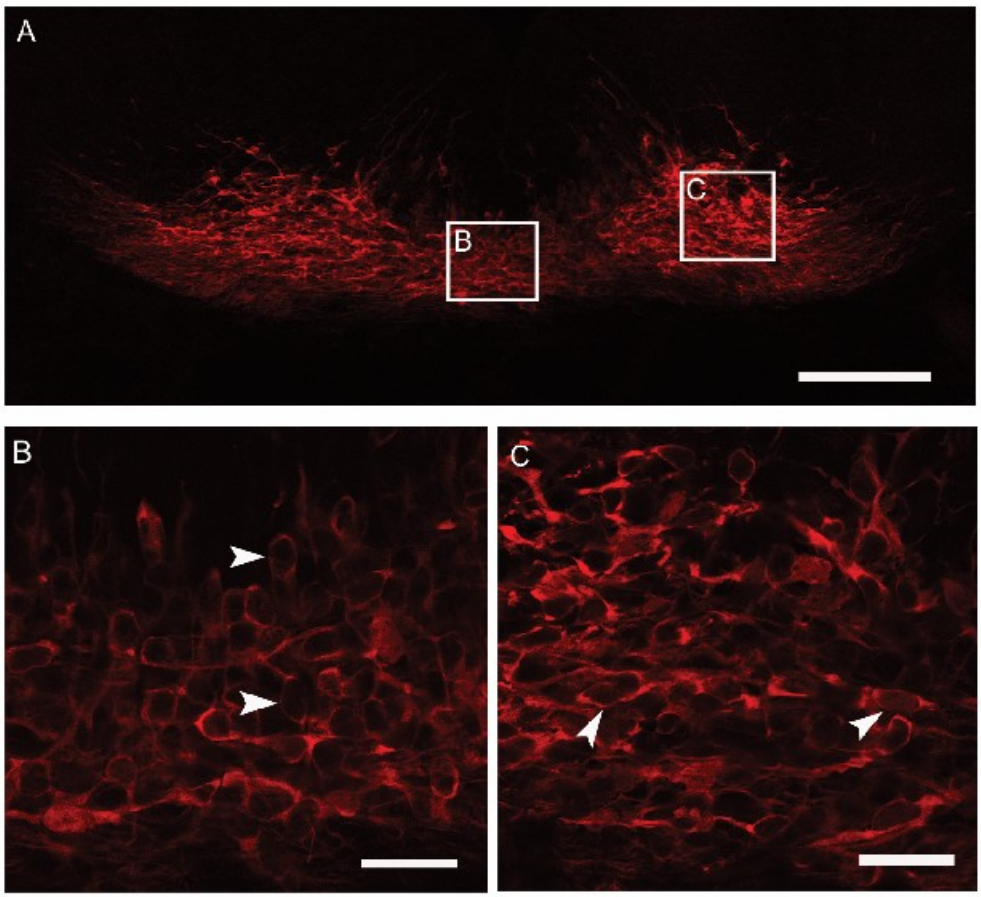

Figure 4.5. Migration pattern of E12.5 mDA neurons.

Sections from E12.5 midbrain were immunostained for TH. Boxed areas in panel (A) at higher magnification show the medial DA cells displaying vertical orientation (B) and the lateral DA cells displaying horizontal orientation (C). Scale bars are 100 µm for panel (A) and 25 µm for panels (B) and (C).

Chapter 5

Phenotype of *Pitx3* knockout mice in development and adulthood

5.1. Introduction

A valuable approach to understanding the functional significance of a gene is to generate mice which lack the gene product and analyse their phenotype. As described in Ch. 3.1, mice have been generated in which the entire *Pitx3* coding sequence has been replaced by a GFP reporter via homologous recombination (Zhao et al., 2004). In *Pitx3* heterozygous mice the *Pitx3*-GFP reporter was used to confirm the mDA neuron restricted expression of Pitx3 in the CNS and reveal that virtually all adult SNc and VTA mDA neurons express Pitx3 (Ch. 3.2). The specific expression of Pitx3 in mDA neurons in the CNS and the fact that it is a homeodomain containing transcription factor make Pitx3 a potential candidate to play a role in mDA neuron development. To investigate this potential role of Pitx3, mice homozygous for the *Pitx3*-GFP mutation (i.e. *Pitx3* null) have been bred and they are viable and fertile. The only obvious abnormality with the *Pitx3* null mice is that they have no eyes due to the crucial role of Pitx3 in lens development (Rieger et al., 2001; Semina et al., 2000). In order to investigate the mDA neuron phenotype of the *Pitx3* null mice three stages of development were studied in detail: E12.5 was the earliest stage looked at as this was the first time point at which Pitx3 protein could be detected. The next stage examined was 2 days later at E14.5, at this stage the structure formed by the mDA cells is recognisable as the SNc and VTA primordium. Finally, the adult midbrain of *Pitx3* null mice was assessed.

During the course of this study a number of papers were published describing the phenotype of the naturally occurring mouse mutant *aphakia* (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). *Aphakia* is a spontaneously occurring recessive phenotype that is associated with a double genomic deletion in the *Pitx3* gene. The *aphakia* homozygous mouse was characterised by small eyes with no lens due to lack of lens development beyond E11, the lens stalk stage (Varnum and Stevens, 1968; Zwaan, 1975). More recently, a deletion in the *Pitx3* promoter region (Semina et al., 2000) and a deletion

in the gene itself, eliminating the non-coding exon 1 and part of intron 1 (Rieger et al., 2001) have been identified as the cause of abnormal ocular development in the *aphakia* mouse. *Aphakia* mice have a 652bp deletion 2.5kb upstream from the putative *Pitx3* transcription start site and a 1423bp deletion that removes non-coding exon 1 and the putative *Pitx3* promoter region in intron 1 (Rieger et al., 2001; Semina et al., 2000). The studies on the midbrain phenotype of *aphakia* mice have shown that adult *aphakia* mice have a loss of TH-expressing cells in the SNc, whereas the VTA is relatively intact. The striatum of *aphakia* mice also displayed a loss of TH immunoreactivity and this was restricted to the dorsal striatum, which is the target structure for projections from the SNc (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). Examination of earlier stages in mDA development of *aphakia* mice has lead to conflicting results. One study reported an altered distribution of TH-expressing cells in E12.5 *aphakia* mice, although the total number of TH-expressing cells does not appear to be reduced (Smidt et al., 2004). However, another study found no changes in TH expression in E12.5 *aphakia* midbrain as compared to wild type (Van Den Munckhof et al., 2003). These results indicate that Pitx3 does have a role in mDA neuron development, especially in the SNc. However, the discrepancies between the *aphakia* studies as to the timing and localisation of the deficit in mDA neurons along with the lack of quantitative data make it difficult to derive the true function of Pitx3 in mDA neurons. Additionally, as the *aphakia* mutation affects the promoter regions of the *Pitx3* gene and the entire coding sequence of *Pitx3* remains intact, it is unclear whether the phenotype of the *aphakia* mice fully reflects the requirement for Pitx3 in mDA neuron development. Consequently, a study of a true *Pitx3* null mouse line with a deletion of the entire Pitx3 coding sequence is required to evaluate the function of Pitx3 in mDA neurons.

5.2. Results

5.2.1. Nascent mDA neurons are absent in *Pitx3* null mice

Firstly, the numbers of TH^+ neurons in E12.5 *Pitx3* heterozygous and wild-type embryos were compared, by counting the numbers of TH^+ neurons in serial sections from 5 embryos of each genotype. The average number of TH^+ neurons in *Pitx3* heterozygous and wild-type embryos were very similar at 1814±469 and 1870±293, respectively (Table 5.1). This result and observations in adult *Pitx3* heterozygous

mice (see below) indicate that the heterozygous mutation does not affect mDA neuron development or survival. Thus *Pitx3* heterozygous mice can be used as a control allowing the GFP reporter to be exploited in determining the fate of the *Pitx3*-GFP expressing neurons in *Pitx3* null mice.

As described previously in Ch. 4.2.1 the *Pitx3* heterozygous E12.5 mouse mesencephalon contains cells expressing both Pitx3 and TH as well as cells that express only one of these markers (i.e. *Pitx3*-GFP^{+}TH^{-} and *Pitx3*-GFP^{+}TH^{-} cells) (Ch. 4.2). Quantitative analysis of E12.5 *Pitx3* heterozygous and *Pitx3* null embryos was performed by counting all labelled cells in serial coronal sections (20µm thick) of an entire midbrain (Fig. 5.1). Total counts from four or five E12.5 embryos of each genotype were averaged and standard deviations were calculated (see Ch. 2.2.7). This revealed that there was no significant difference in the number of *Pitx3*-GFP^{+}TH^{-} cells at E12.5 (Table 5.1) suggesting that Pitx3 is not required for the generation or maintenance of the *Pitx3*-GFP^{+}TH^{-} mDA neuron progenitors. However, there was a loss of around half of the *Pitx3*-GFP^{+}TH^{+} cells in the E12.5 *Pitx3* null mice (628±362) as compared with *Pitx3* heterozygous (1211 ± 316) (Table 5.1). Also there was a reduced number of total mDA neurons in E12.5 *Pitx3* null mice (834 ± 419) compared to Pitx3 heterozygous mice (1814±469) and a significant decrease in the number of *Pitx3*-GFP^{-}TH^{+} cells in the *Pitx3* null (237±126) as compared to *Pitx3* heterozygous mice (605 ± 234) (Table 5.1). Since it is the total TH^{+} cells (both the *Pitx3*-GFP^{-}TH^{+} and *Pitx3*-GFP^{+}TH^{+} cells) rather than the *Pitx3*-GFP^{+}TH^{-} cells that are lost in the *Pitx3* null mice, this data indicates that Pitx3 is necessary for the generation or the maintenance of nascent mDA neurons.

5.2.2. Pitx3 is required for TH expression in SNc neurons

The lack of TH expression in only some *Pitx3*-GFP expressing cells in the *Pitx3* null mice at E12.5 suggests that Pitx3 is not necessary for correct TH expression in all mDA neurons, but that the requirement for this function of Pitx3 may display cell type-specificity. If this is the situation then it would be expected that the deficiency in TH expression would be restricted to particular sub-regions of the *Pitx3* null midbrain. To investigate this, E14.5 brains were studied as at this stage the VTA and SNc primordium become recognisable.

In contrast to the situation at E12.5, by E14.5 the majority of mDA cells are Pitx3^{+}TH^{+}. E14.5 midbrain sections double stained with antibodies against TH and GFP revealed a specific loss of TH expression in the developing SNc of the *Pitx3* null mice when compared to *Pitx3* heterozygous mice (Fig. 5.2). Quantitative analysis showed that the loss of TH expression in *Pitx3*-GFP cells was restricted to the SNc, with 53 ± 8% of *Pitx3*-GFP cells expressing TH in *Pitx3* null mice compared to 88 ± 5% in *Pitx3* heterozygous mice (Fig 5.3), whereas the percentage of *Pitx3*-GFP cells expressing TH in the VTA was approximately the same in *Pitx3* null (84 ± 17%) and *Pitx3* heterozygous (81 ± 14%) mice (Fig 5.3). As expected from the deficiency in TH expression in the SNc of *Pitx3* null mice, there were a greater number of *Pitx3*-GFP^{+}TH^{-} cells in the SNc of *Pitx3* null (519±15.3) as compared to *Pitx3* heterozygous (189±7.9) (Table 5.1, Figure 5.2). The total midbrain counts of *Pitx3*-GFP expressing and TH expressing cells revealed that there was no significant difference in the numbers of *Pitx3*-GFP^{-}TH^{+} cells or *Pitx3*-GFP^{+}TH^{+} cells between *Pitx3* heterozygous and *Pitx3* null mice (Table 5.1). It is likely that the large numbers of VTA cells mask the SNc effect on the cell numbers. However, from the total midbrain counts it was possible to detect significantly higher numbers of *Pitx3*-GFP^{+}TH^{-} cells in *Pitx3* null mice (6773±1356) as compared to their heterozygous littermates (1834±341) (Table 5.1). This data demonstrates the requirement for Pitx3 for the maintenance and/or initiation of TH expression in a subset of mDA neurons.

5.2.3. The loss of mDA neurons is restricted to SNc neurons

As well as a lack of TH expression, there was a deficiency of over half of the *Pitx3*-GFP^{+}TH^{+} cells in the SNc of the *Pitx3* null (596±11.3) as compared to *Pitx3* heterozygous littermates (1415±43.7) (Table 5.1). Additionally, to a much lesser degree than in the SNc, there was some loss of *Pitx3*-GFP^{+}TH^{+} cells in the VTA of the *Pitx3* null mice (813±19.9) as compared to heterozygous littermates (1161±25.3) (Table 5.1). This indicates that Pitx3 is required for the survival and/or maintenance of Pitx3^{+}TH^{+} cells in the SNc.

These results reveal that two elements likely contribute to the reduction in the number of DA neurons (TH expressing cells) in the SNc of E14.5 *Pitx3* null mice. One element is the failure of TH expression in some *Pitx3*-GFP expressing cells and the other is the loss of *Pitx3*-GFP^{+}TH^{+} cells.

Age	Genotype	GFP^+TH^-	GFP^-TH^+	GFP^+TH^+	Total TH^+
E12.5	Pitx3 wt (n=4)	n/a	n/a	n/a	1870±293
	Pitx3+/- (n=5)	608±378	605±234	1211±316	1814±469
	Pitx3-/- (n=5)	809±264	237±126	628±362	834±419
	P<=	0.35[a]	0.027[a]	0.029[a]	0.84[b]
E14.5 total	Pitx3+/- (n=3)	1834±341	4364±629	17622±4397	
	Pitx3-/- (n=3)	6773±1356	4068±279	14175±3600	
	P<=	0.004	1	1	
E14.5 SNc	Pitx3+/- (n=3)	189±7.9	211±25.8	1415±43.7	
	Pitx3-/- (n=3)	519±15.3	108±5.7	596±11.3	
	P<=	$2.733x10^{-5}$	0.2331	$4.363x10^{-5}$	
E14.5 VTA	Pitx3+/- (n=3)	272±29.8	123±15	1161±25.3	
	Pitx3-/- (n=3)	150±16.1	160±10.7	813±19.9	
	P<=	0.2698	0.5344	0.003417	

Table 5.1. Quantitative analysis of E12.5 and E14.5 mesencephalic DA cells in wildtype, *Pitx3* heterozygous and *Pitx3* null mice

E12.5 and E14.5 total results were obtained from the counting of all labelled cells in serial sections from three embryos for each genotype, and the numbers of cells from each section were summed. E14.5 SNc and VTA results were obtained from five midbrain sections from three embryos for each genotype.

(a, Student *t*-test between *Pitx3*+/- and *Pitx3*-/-; *b*, Student *t*-test between *Pitx3* wt and *Pitx3*+/-; n/a, not applicable; n, number of embryos)

5.2.4. Progressive loss of mDA neurons results in the absence of SNc mDA neurons in *Pitx3* null adult mice

Adult midbrain and striatum were immunostained with antibodies against TH alone and this showed that *Pitx3* heterozygous and wild type littermates have a comparable number of TH expressing cells, which, along with the data from E12.5 mice (Table 5.1), confirms that mDA neurons in *Pitx3* heterozygous mice appear to be phenotypically normal. However, in the *Pitx3* null midbrain there is a considerable loss of TH expressing cells in the SNc, whilst the VTA is relatively intact (Fig. 5.4). This implies that the loss of mDA neurons observed in the SNc at E14.5 progresses to result in a complete loss of mDA neurons in adulthood. Examination of *Pitx3* heterozygous and *Pitx3* null midbrains for *Pitx3*-GFP and TH expression revealed

that the loss of TH expression was mirrored by a loss of *Pitx3*-GFP expression in *Pitx3* null mice, resulting in a complete loss of *Pitx3*-GFP$^+$TH$^+$ cells in the SNc (Fig. 5.5). Although the possibility that Pitx3 is required to regulate its own expression cannot be ruled out, the fact that both TH and *Pitx3*-GFP expressing cells are lost in the SNc suggests that these cells actually die rather than just down-regulate TH and Pitx3 expression. Further evidence for cell loss is presented in Chapter 6.

Axons from the SNc and VTA project to different target structures in the striatum. The SNc mDA neurons project to the dorsolateral striatum known as the caudate putamen, whereas the VTA mDA neurons project to the ventromedial striatum, nucleus accumbens and olfactory tubercle. The mDA neuron afferents in the striatum are the major source of TH immunoreactivity in the striatal target areas of the VTA and SNc neurons. The existence of intrinsic TH-positive neurons in the striatum has been reported, however, the presence of these cells is controversial and if valid there are likely to be relatively few cells (Baker et al., 2003; Cossette et al., 2004). To determine whether the mDA projections from the SNc and VTA are intact and innervating the correct areas in *Pitx3* null mice, the striatal DA afferents were examined for the presence of TH. In the *Pitx3* heterozygous and wild type striatum TH antibody staining is present throughout the caudate putamen, nucleus accumbens and olfactory tubercle (Fig. 5.4). However in the *Pitx3* null striatum there was a lack of TH antibody staining in the dorsal caudate putamen, which is the target innervation area for SNc neurons. Yet in the VTA target areas, which include the ventral caudate putamen, nucleus accumbens and olfactory tubercle, TH staining was present in *Pitx3* null mice. This confirms that the VTA neurons and their striatal projections remain intact and innervate the correct targets, whereas the SNc neurons and their afferents are lost or do not contain TH in *Pitx3* null mice.

5.3. Summary

These studies have demonstrated that there is a progressive loss of mDA neurons in *Pitx3* null mice which is restricted to the SNc. At E12.5 there is a 50% loss of *Pitx3*-GFP$^+$TH$^+$ cells, however, at this stage it was not possible to determine if these were SNc or VTA cells. By E14.5 there is around a 60% loss of *Pitx3*-GFP$^+$TH$^+$ cells in the SNc of *Pitx3* null mice. Eventually, by adulthood there is a complete loss of *Pitx3*-

GFP^+TH^+ neurons in the SNc of *Pitx3* null mice. The fact that there are no TH or *Pitx3*-GFP expressing cells in the adult SNc indicates that the cells are actually lost rather than just down-regulated TH expression. However, there is also the possibility that the cells remain but have down-regulated both TH and Pitx3 expression and further evidence that these cells are actually lost via apoptosis is provided in chapter 6. In addition to the loss of *Pitx3*-GFP^+TH^+ cells, during embryonic development there was a specific loss of TH expression in some *Pitx3*-GFP cells in *Pitx3* null mice resulting in an increased number of *Pitx3*-GFP^+TH^- cells. At E12.5 there was a significant decrease in the number of *Pitx3*-GFP positive cells expressing TH in the *Pitx3* null mice as compared to *Pitx3* heterozygous. Furthermore, there was a loss of TH expression in *Pitx3*-GFP cells at E14.5 in *Pitx3* null mice and by this stage it was clear that this loss of TH expression was restricted to the SNc. These data demonstrate that Pitx3 is not only required for the survival of mDA neurons but it is also involved in the regulation of TH expression in a subset of mDA neurons.

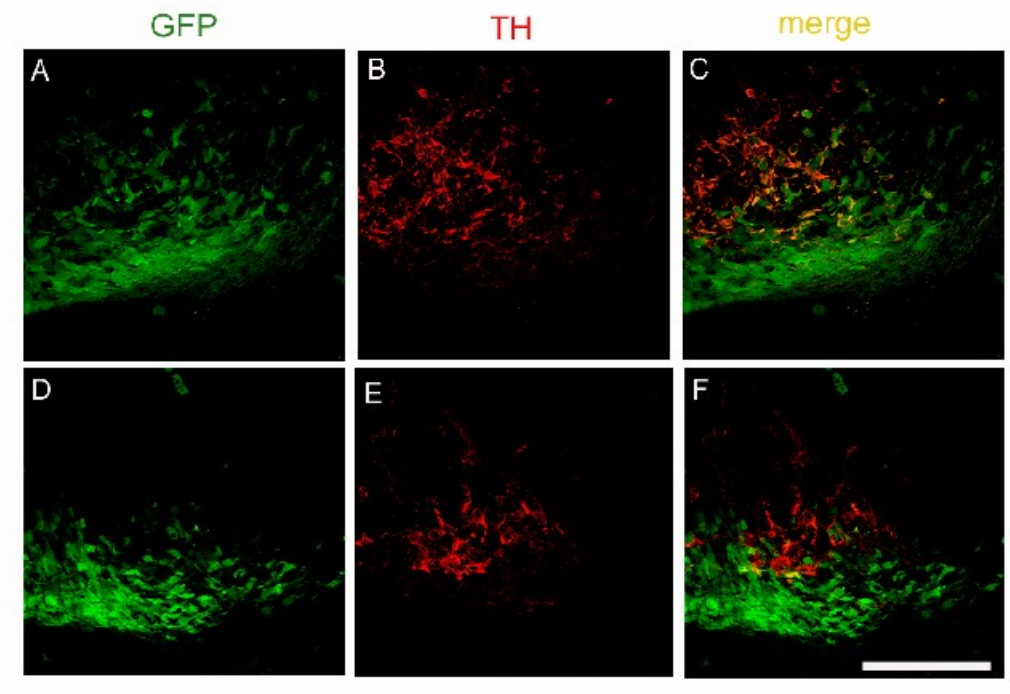

Figure 5.1. Analysis of *Pitx3* and TH expression in the E12.5 midbrain of *Pitx3* null mice.

E12.5 Pitx3 heterozygous and Pitx3 null 20µm coronal midbrain sections stained with antibodies against GFP and TH. There are a reduced number of TH labelled cells in the *Pitx3* null (A-C) as compared to the *Pitx3* heterozygous (D-F). Scale bar 100µm.

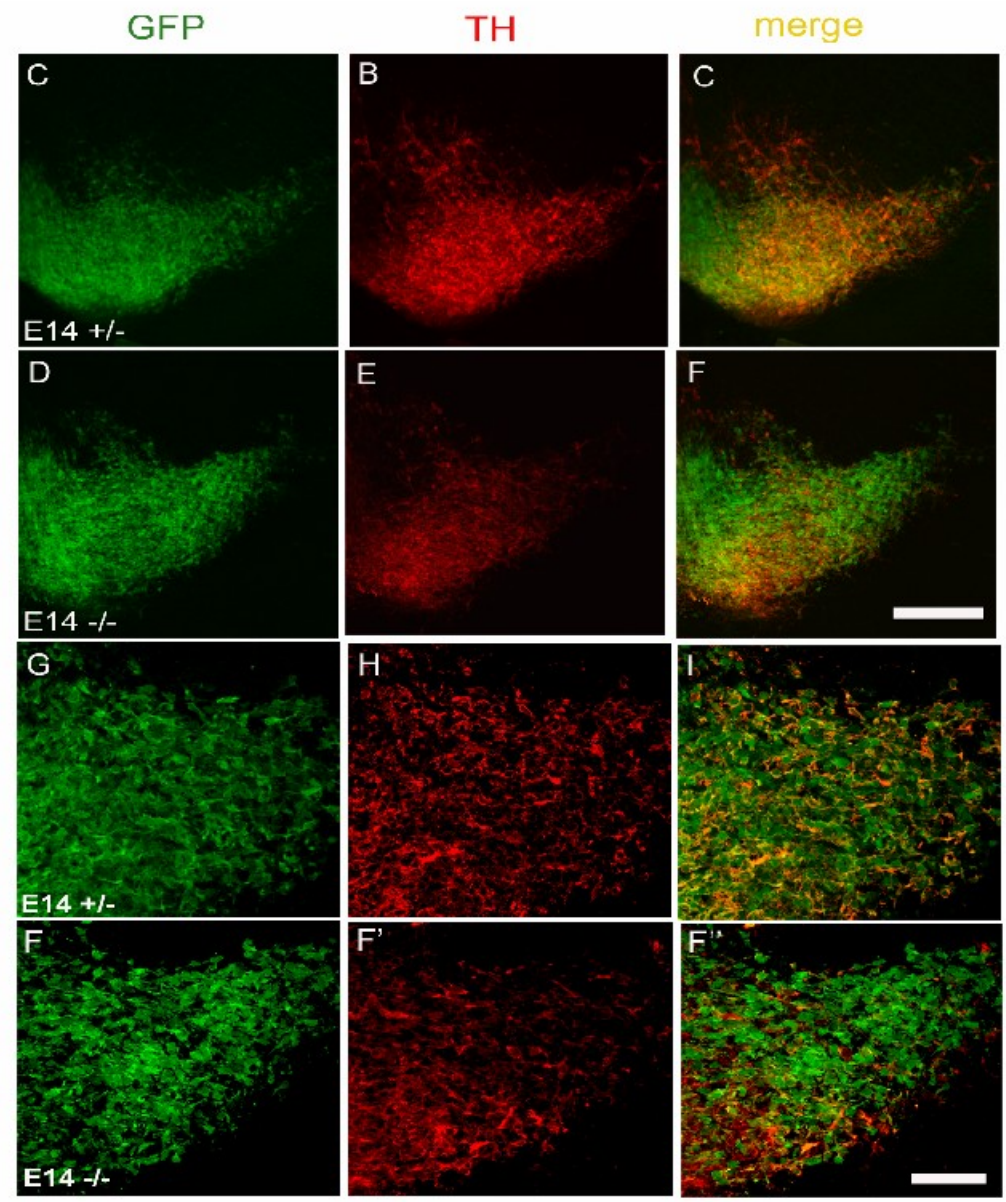

Figure 5.2. Analysis of *Pitx3* and TH expression in the E14.5 midbrain of *Pitx3* null mice.

GFP and TH antibody stained E14.5 20µm coronal sections show that the loss of TH^+ cells is restricted to the SNc in the *Pitx3* null (D-F) as compared to the *Pitx3* heterozygous (A-C). Higher magnification images of the SNc of *Pitx3* heterozygous (G-I) and *Pitx3* null (J-L). Scale bar 200µm for A-F and 50 µm for G-L.

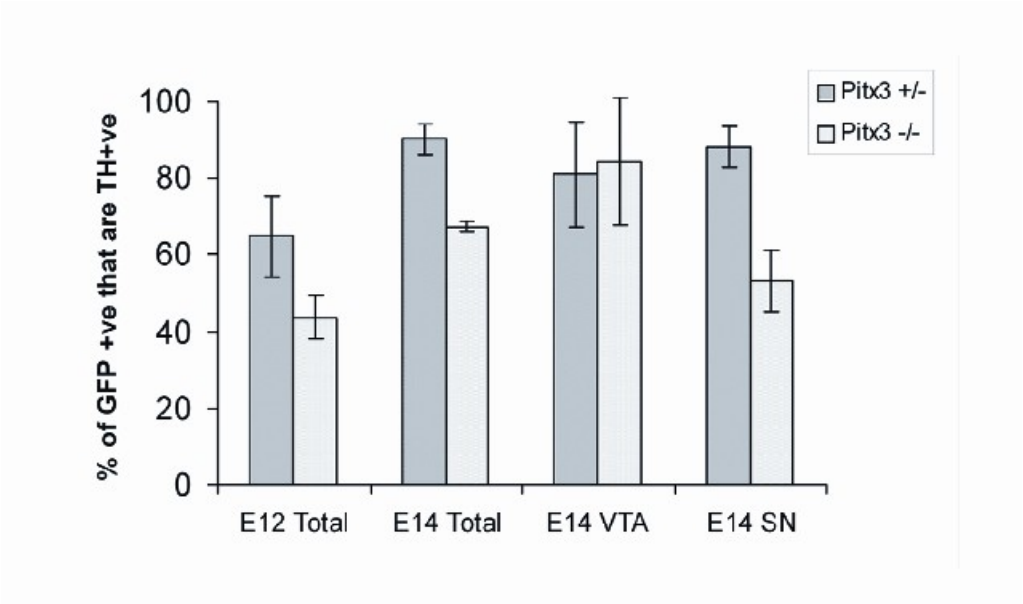

Figure 5.3. Pitx3 regulates TH expression specifically in the SNc mDA neurons. Quantitative analyses showing the percentage of GFP-expressing cells that also express TH. E12.5 and E14.5 total results were obtained from the counting of all labelled cells in serial sections for three embryos from each genotype. E14.5 SNc and VTA results were obtained from five sample midbrain sections from three embryos from each genotype.

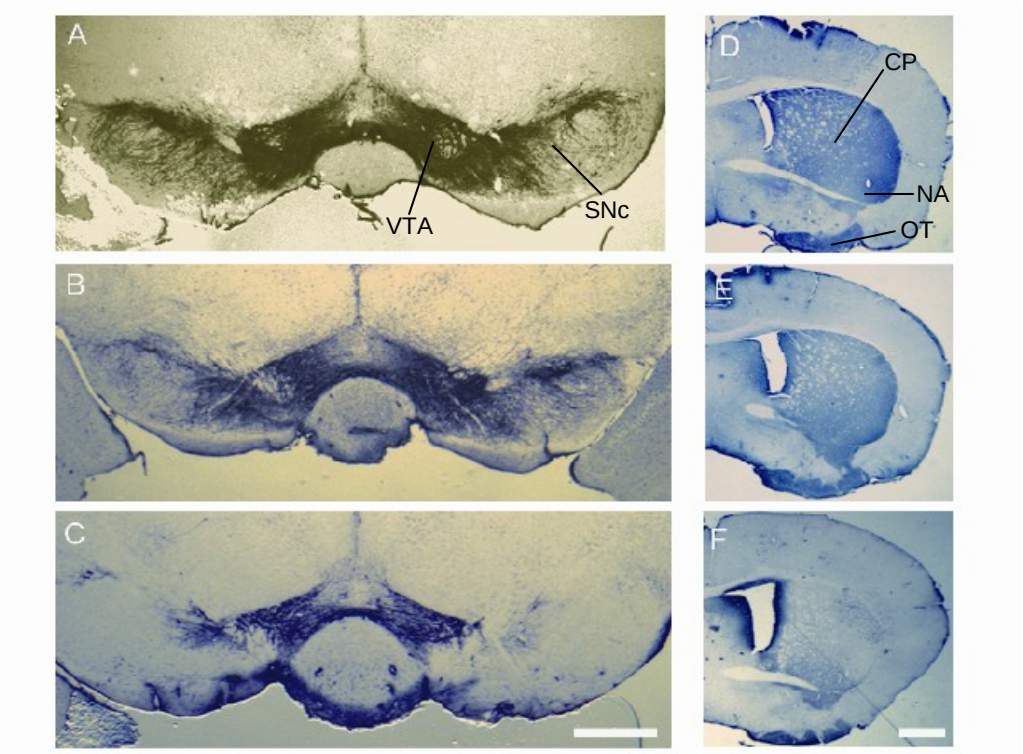

Figure 5.4. Analysis of TH expression in the adult midbrain of *Pitx3* null mice. Immunostaining for TH in coronal sections of wild type, *Pitx3* heterozygous and *Pitx3* null adult mice brains reveals that the wild type and Pitx3 heterozygous midbrains are comparable and display TH expression in SNc and VTA neurons (A and B). Also, the striatal areas of wild type and *Pitx3* heterozygous mice have TH-immunostaining throughout the caudate putamen (CP), nucleus accumbens (NA) and olfactory tubercle (OT) (D and E). However, the Pitx3 null mice have a complete loss of TH expressing cells in the SNc while the TH expression in the VTA remains intact (C). Accordingly the target area of the SNc the dorsal caudate putamen lacks TH immunostaining, while TH-immunostaining in the VTA target areas the ventral caudate putamen, nucleus accumbens and olfactory tubercle is present (F). Scale bars 1mm.

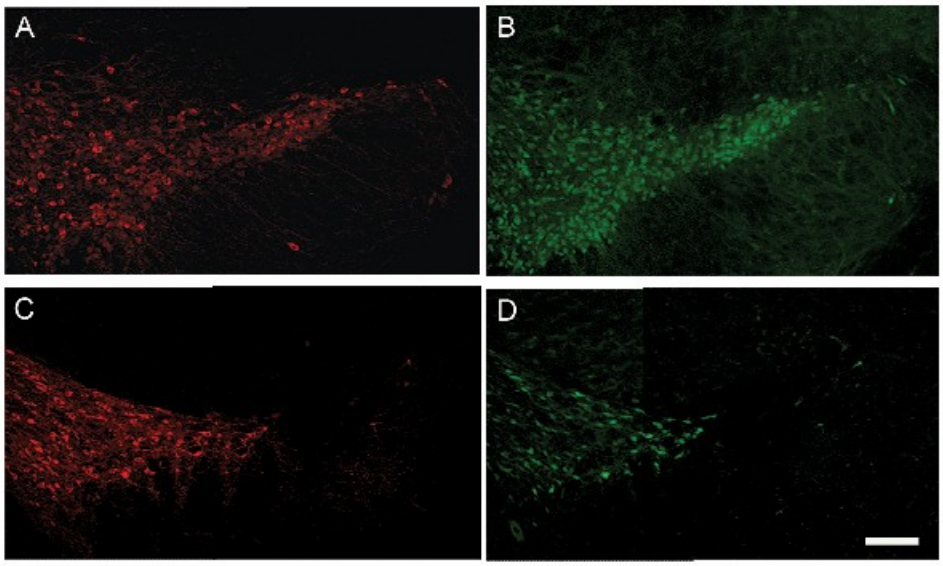

Figure 5.5. Analysis of *Pitx3* and TH expression in the adult midbrain of *Pitx3* null mice.

Coronal midbrain sections double immunolabelled with antibodies against GFP and TH show that there is a loss of both *Pitx3*-GFP and TH expressing cells in the SNc of *Pitx3* null mice (C, D) as compared to Pitx3 heterozygous mice (A, B), whereas *Pitx3*-GFP and TH expressing cells remain in the VTA of Pitx3 null mice (C, D). Scale bar 300µm.

Chapter 6

Mechanism of cell loss in midbrain dopaminergic neurons of *Pitx3* null mice

6.1. Introduction

In the SNc of *Pitx3* null mice there is a deficit of *Pitx3*-GFP$^+$ and TH$^+$ cells (Chapter 5). The loss of *Pitx3*-GFP$^+$ and TH$^+$ cells appears to be gradual throughout development with a reduction in the number of SNc *Pitx3*-GFP$^+$TH$^+$ cells by approximately 50% at E12.5 and 60% at E14.5 to a complete loss by adulthood. From the results obtained in chapter 5, it was not possible to ascertain whether the *Pitx3*-GFP$^+$ and TH$^+$ cells in the SNc of *Pitx3* null mice were dying or not. Potentially these cells may still be present but have simply down-regulated expression of Pitx3 and TH. Another possibility is that they may have down-regulated expression of Pitx3 and TH and changed fate to become another neuronal subtype. To elucidate this situation a quantitative and qualitative assessment of cell death in the SNc was undertaken. To assess the likelihood of a cell fate change in the *Pitx3* null SNc mDA neurons, further marker analysis was performed on these cells. In addition, a chimera study was carried out to identify whether the fate of the *Pitx3* null SNc mDA cells could be rescued by any extracellular factors.

6.2. Results

6.2.1. SNc mDA cells are lost via apoptosis in *Pitx3* null midbrain

To investigate whether there is evidence of increased apoptosis in vivo and to allow the region where apoptosis occurs to be identified, sections from E14.5 and newborn mice were examined for evidence of cell death via apoptosis.

6.2.1.1. TUNEL labelling in E14.5 midbrain sections

Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labelling (TUNEL) allows the marking of cells that are undergoing apoptotic cell death. The TUNEL process labels the free 3'-OH ends created by internucleosomal DNA fragmentation, which occurs during apoptosis, with digoxygenin labelled nucleotides which can then be detected with antibodies. TUNEL was performed on E14.5 midbrain sections along

with GFP antibody staining. Quantitative analysis revealed that there was an increase in apoptotic cells in the *Pitx3*-GFP cells of *Pitx3* null embryos as compared to Pitx3 heterozygous and this was restricted to the SNc (Fig. 6.1). The number of apoptotic cells is around 1% of *Pitx3*-GFP$^+$ cells in the SNc (1.2 ± 0.3%) and VTA (1.1 ± 0.4%) of *Pitx3* heterozygous and the VTA (1.2 ± 0.2%) of *Pitx3* null although it increases by around 1% to 2.4 ± 0.3% in the SNc of *Pitx3* null embryos (Fig. 6.1). An increase of 1% may seem negligible, however, as the TUNEL technique labels the execution phase of apoptosis, which in cell culture takes less than 6 hours, a small percentage of TUNEL positive cells can result in a great increase in cell death. For example, a degree of tissue degeneration of 25% per day can result from an apparent apoptosis of 2-3% of cells at any instant (Bursch et al., 1990).

6.2.1.2. Nissl staining on newborn brain sections

If cell death, rather than just the loss of dopaminergic cell phenotype, has occurred in the SNc of *Pitx3* null mice then a decreased cellularity in the *Pitx3* null SNc would be expected. Midbrain sections from newborn brains were Nissl stained to reveal the neuronal density and morphology in *Pitx3* heterozygous and *Pitx3* null mice. This showed that there were much fewer neurons and decreased cellularity in the SNc of *Pitx3* null midbrain compared to *Pitx3* heterozygotes (Fig. 6.2). Furthermore, many of the cells remaining in the SNc of *Pitx3* null mice were dead and displayed highly condensed pyknotic nuclei, a characteristic feature of apoptosis.

6.2.2. Increased apoptosis of E14.5 *Pitx3* null cells in primary culture

The TUNEL assay revealed that in E14.5 *Pitx3* null midbrain SNc cells were dying due to apoptosis. To investigate whether *Pitx3* null cells can be rescued or continue to die via apoptosis when isolated from the midbrain environment they were cultured in an environment favouring DA neuron production. It has been reported that co-culture of ES cells with PA6 cells results in efficient neuronal differentiation with a high proportion of TH-expressing neurons (Kawasaki et al., 2000). Thus, primary mDA neurons were cultured on a layer of PA6 cells to support DA neuron differentiation and survival.

Midbrains from *Pitx3* heterozygous and *Pitx3* null E14.5 embryos were dissected using the *Pitx3*-GFP marker as a guide to isolate the mDA cells in the ventral midbrain. Midbrain tissue was dissociated manually to a single cell suspension and cultured on a layer of PA6 cells for 24 hours before staining for antibodies against the

activated form of caspase-3 and GFP (Ch. 2.3.1). Caspase-3 is a major effector of neuronal apoptosis and can be induced by a variety of stimuli. This cell death protease undergoes cleavage to become activated before it catalyses the specific cleavage of many key cellular proteins. Quantitative analysis revealed a significant increase in the number of activated caspase-3 positive cells in the *Pitx3*-GFP cell population in the Pitx3 null cultures (6.0 ± 2.6%) as compared to the Pitx3 heterozygous cultures (3.2 ± 1.7%) (Fig. 6.3).

The rate of apoptotic cell death in the *Pitx3* null mDA cells is around two-fold higher than in *Pitx3* heterozygous mDA cells in this PA6 co-culture system, which is a similar fold increase to that observed by TUNEL labelling in midbrain sections (Ch 6.2.1.1). This suggests that the factors provided by the PA6 cells and culture media cannot rescue the fate of the *Pitx3* null mDA cells.

6.2.3. Is there a cell fate change for *Pitx3* null mDA cells?

The progressive restriction of neural cell fate involves the interplay of both extrinsic and intrinsic signals and may occur at many different stages and times during development (Edlund and Jessell, 1999). Transplantation experiments may be used to assess the degree of cell fate commitment to certain neuronal subtypes at different stages of development. Transplanting En1 expressing midbrain cells to the forebrain revealed that at E10.5 the majority of these cells can be respecified to a forebrain phenotype, however at E13.5 the majority of transplanted cells do not integrate and still express midbrain markers (Olsson et al., 1997). As En1 is expressed in mDA progenitors at E10.5 and mDA neurons at E13.5, these studies suggest that mDA progenitors become restricted in their potential between E10.5 and E13.5.

The experiments above show that at least some of the *Pitx3* null SNc mDA cells are lost due to apoptotic cell death. To investigate the possibility that some *Pitx3* null cells may undergo a fate change, the expression of the mDA marker En1 and the GABAergic neuron marker GAD were assessed in E12.5 and E14.5 *Pitx3* null midbrains. Analysis of GABAergic neurons was selected because the SNc and VTA of adult mice contains GABAergic neurons as well as mDA neurons [Sarabi, 2001 #151;Michel, 2004 #150], however, little is known about the developmental expression of GAD in the midbrain. At E12.5 and E14.5, *Pitx3*-GFP cells in the *Pitx3* null midbrain expressed En1 (Fig. 6.4, G-L), which suggests that these cells are mDA phenotype rather than any other neuron type. In concurrence, the anti-GAD antibody labelled cells dorsal to the mDA neuron domain which was distinct from the *Pitx3*-

GFP expressing domain (Fig. 6.4, A-F), indicating that all *Pitx3* expressing cells at E12.5 and E14.5 are not GABAergic neurons. However, the possibility that *Pitx3* null cells may change fate accompanied with a downregulation of *Pitx3*-GFP expression cannot be ruled out.

6.2.4. Pitx3 acts in a cell autonomous manner

To investigate whether Pitx3 or any of its downstream targets were able to act in a cell non-autonomous manner and rescue *Pitx3* null cells in the SNc, a chimera study was performed. Chimeric embryos were generated from either *Pitx3* heterozygous or *Pitx3* null ES cells with wild type host embryos using the diploid aggregation method (Ch. 2.4.5) (all aggregations and embryo transfers were performed by R. MacLay, G. Russell, and J. Agnew at the ISCR). The chimeras produced displayed varying degrees of chimerism and some had such a great extent of contribution from the *Pitx3*-GFP cells that they resembled the non-chimeric *Pitx3* heterozygous or *Pitx3* null midbrain phenotype. In this situation there is not likely to be a competition effect between wild type and *Pitx3*-GFP cells, so lower level chimeras (approximately 30-70% *Pitx3*-GFP cell contribution) were used for analysis. Sections from the midbrains of E14.5 chimeric mice were analysed using antibodies against GFP to detect the *Pitx3* heterozygous or *Pitx3* null cells and TH to reveal all the mDA neurons and therefore reveal the SNc and VTA structures. In chimeric mice made with *Pitx3* heterozygous ES cells the *Pitx3*-GFP cells contributed to both the VTA and the SNc areas (Fig 6.6). On the other hand, in chimeric mice made with *Pitx3* null ES cells the *Pitx3*-GFP cells showed contribution to the VTA but rarely to the SNc (Fig 6.6). As the level of chimerism in each embryonic chimeric mouse was different, the quantitative analysis could not be based on absolute numbers. Instead, the ratio of the number of *Pitx3*-GFP cells in the SNc to the number of *Pitx3*-GFP cells in the VTA was compared between chimeras. The VTA acts as a control region where no variation is expected between the *Pitx3* heterozygous and the *Pitx3* null ES cell contribution as the VTA phenotype is not significantly different in *Pitx3* heterozygous and *Pitx3* null E14.5 embryos (Ch. 5.2). This demonstrated quantitatively that the *Pitx3* null cells were impaired in contributing to the SNc, as the ratio of SNc:VTA *Pitx3*-GFP cells in the chimeras made with *Pitx3* null ES cells (0.017 ± 0.01) was much lower than in the chimeras made with *Pitx3* heterozygous ES cells (0.22 ± 0.05) (Fig. 6.6). The interpretation of this experiment is limited, however, by the fact that the donor *Pitx3*-GFP ES cells can only be detected by virtue of their *Pitx3*-GFP expression. As there is no constitutive marker for the donor ES cell-derived cells in

this system, *Pitx3*-GFP cells which have contributed to the SNc but have downregulated *Pitx3*-GFP expression are therefore undetectable.

As an additional control, the SNc:VTA *Pitx3*-GFP cell number ratios in non-chimeric *Pitx3* heterozygous and *Pitx3* null mice were analysed. If the ES cells used to make chimeras are not impaired in their ability to contribute to the SNc, it would be expected that the SNc:VTA *Pitx3*-GFP cell ratio in the chimeric mice would be the same as the ratio observed in non-chimeric mice. As shown above the Pitx3 null cells are impaired in their ability to contribute to the SNc and as expected the ratio of SNc:VTA *Pitx3*-GFP cells in the *Pitx3* null chimeric mice (0.017 ± 0.01) is much lower than the ratio in the *Pitx3* null mice (1.05 ± 0.22). However, the SNc:VTA *Pitx3*-GFP cell ratio in the chimeric mice made with *Pitx3* heterozygous ES cells (0.22 ± 0.05) is also much lower than the in Pitx3 heterozygous mice (1.38 ± 0.25) (Fig. 6.6). This suggests that the *Pitx3* heterozygous ES cells also have a reduced ability to contribute to the SNc, but this defect is only apparent when these cells are challenged such as in the chimera situation.

6.3. Summary

These studies imply that in *Pitx3* null mice SNc mDA neurons are lost due to apoptosis rather than down-regulation of TH and Pitx3 expression or a change of fate into another cell-type. The TUNEL assay and Nissl staining performed on *Pitx3* null embryonic and neonate midbrains, respectively, have provided evidence of apoptotic cell death restricted to the SNc in *Pitx3* null mice. Further support was given by primary culture studies of embryonic midbrain cells which revealed that there was a two-fold increase in apoptotic cell death in *Pitx3* null mDA cells as compared to *Pitx3* heterozygous mDA cells. It is difficult to fully assess whether there is a change of cell fate of *Pitx3*-GFP cells in *Pitx3* null mice, however, it seems unlikely as embryonic *Pitx3*-GFP cells in *Pitx3* null mice express the dopaminergic neuron marker En1 and do not express GABAergic neuron markers. Additionally, the effect of Pitx3 expression in mDA cells is cell autonomous as chimera studies revealed that wild type mDA cells were unable to rescue the *Pitx3* null SNc cells.

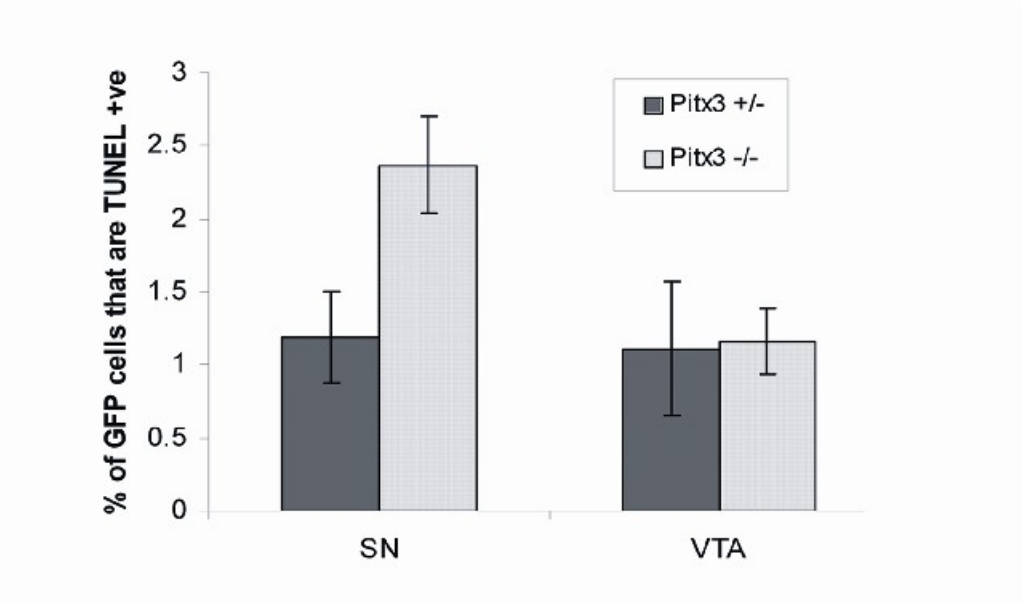

Figure 6.1. TUNEL analysis of E14.5 *Pitx3* heterozygous and *Pitx3* null midbrains.

The bar chart shows the percentage of *Pitx3*-GFP expressing cells that were TUNEL labelled. Midbrain sections from 3 mice were analysed for each genotype. There were significantly more *Pitx3*-GFP^{+} TUNEL labelled cells in the SNc of *Pitx3* null (2.34 ± 0.33%) as compared to *Pitx3* heterozygous (1.18 ± 0.31%), P= 0.01 (student's t-test). Whereas the VTA of the Pitx3 null (1.16 ± 0.23%) and Pitx3 heterozygous (1.11 ± 0.46%) were similar, P= 0.85 (student's t-test).

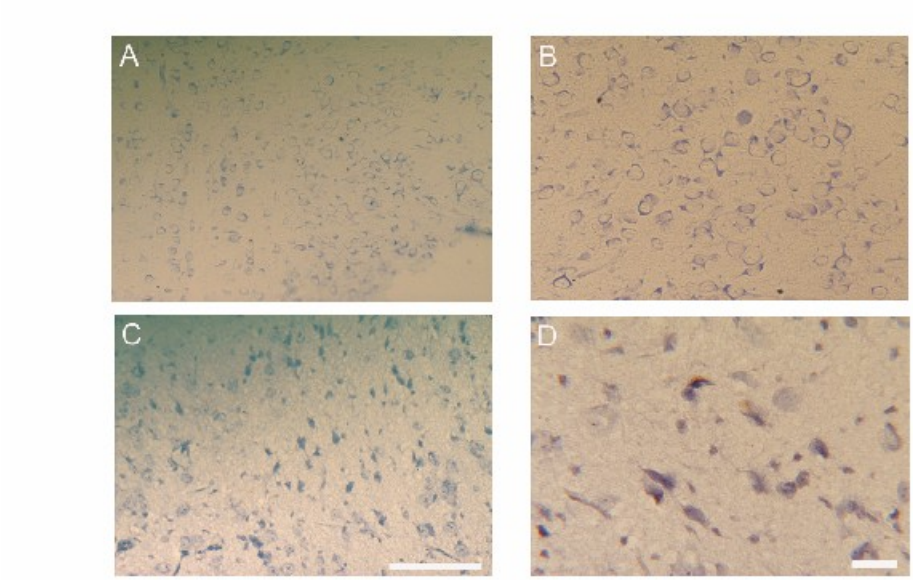

Figure 6.2. Nissl staining on newborn *Pitx3* heterozygous and *Pitx3* null midbrain sections.

Nissl staining was performed on newborn brain sections from *Pitx3* heterozygous (A and B) and *Pitx3* null (C and D) mice. Parallel sections stained with TH antibodies were used to orientate the region of the SNc in the Nissl stained sections. Scale bars 200µm.

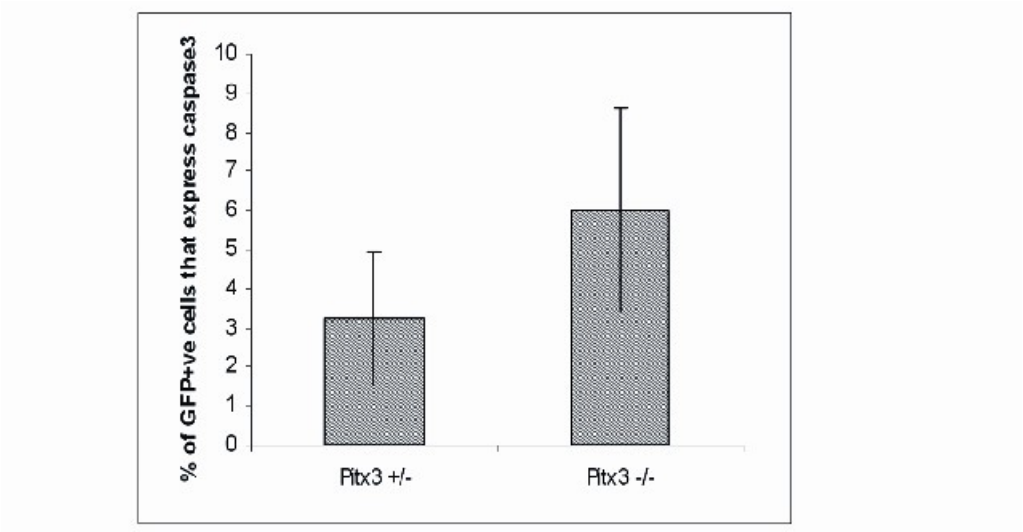

Figure 6.3. Activated caspase-3 expression is increased in *Pitx3* null E14.5 midbrain primary cultures.

The bar graph shows the percentage of *Pitx3*-GFP cells that also expressed activated caspase 3 in primary cultures of *Pitx3* heterozygous (3.2 ± 1.7%) and *Pitx3* null (6.0 ± 2.6%) midbrain cells. For each genotype 15 wells from a total of 3 separate experiments were analysed and on average there were around 150 *Pitx3*-GFP positive cells per well.

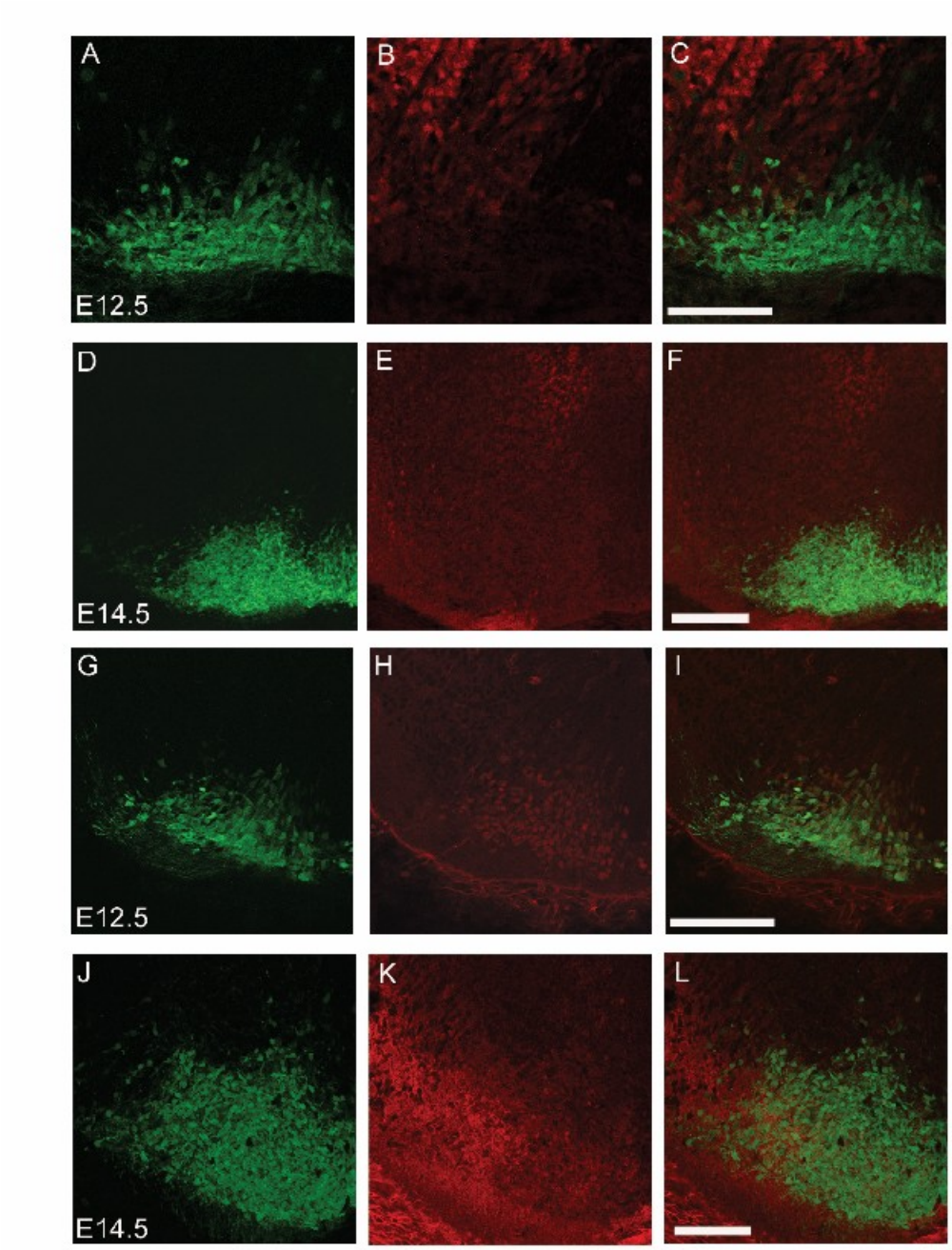

Figure 6.4. Marker analysis in *Pitx3* null midbrains

Coronal sections from E12.5 and E14.5 *Pitx3* null midbrains were stained with antibodies against GAD (red) and GFP (green) (A-F) or En1 (red) and GFP (green) (G-L). The *Pitx3*-GFP cells do not express GAD (A-F) but do express En1 (G-L) at E12.5 and E14.5. Scale bars 100µm for A-C and G-I, 200µm for D-F and J-L.

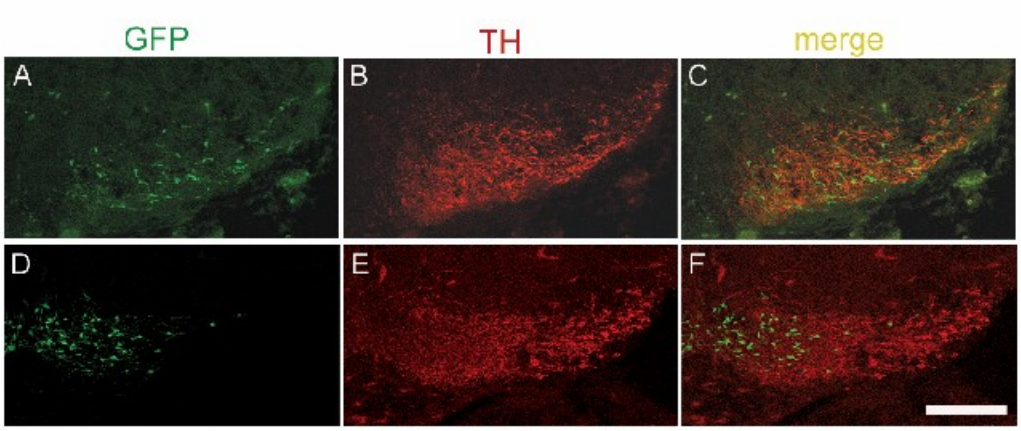

Figure 6.5. Chimeras made with *Pitx3* heterozygous or *Pitx3* null ES cells and wild type embryos

Chimeric E14.5 embryos made with *Pitx3* heterozygous ES cells (A-C) and *Pitx3* null ES cells (D-F) were cryosectioned at 30µm and stained with antibodies against TH and GFP. The mDA neurons in the embryo will be stained with TH and only the cells from either the *Pitx3* heterozygous or null ES cells will be GFP-positive. The *Pitx3* heterozygous cells contributed to both the VTA and SNc (A-C) whereas the *Pitx3* null cells contributed to VTA but not the SNc (D-F). Scale bar 200µm.

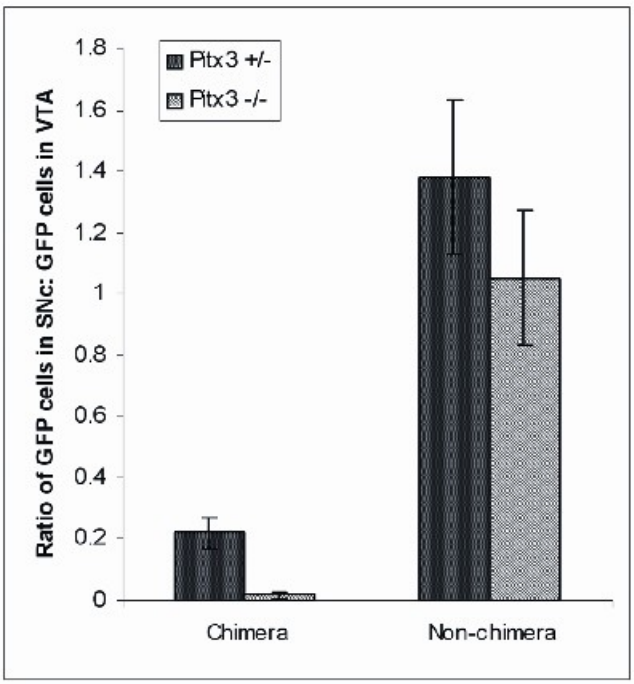

Figure 6.6. Analysis of chimeras made with *Pitx3* heterozygous or *Pitx3* null ES cells and wild type embryos

The chimeric embryo midbrains were analysed by counting all of the *Pitx3*-GFP expressing cells in the SNc and VTA using Volocity software (Improvision). To normalise for differences in the overall level of chimerism, the VTA was used as a control area and the ratio of GFP cells in the SNc:GFP cells in the VTA was calculated. As an additional control, the ratios of GFP cells in the SNc:GFP cells in the VTA from *Pitx3* heterozygous and *Pitx3* null mice were calculated from previous quantitative data (Ch. 5.2). Three chimeric embryos were analysed for each genotype.

Chapter 7

Production of Pitx3-CreER^{T2} ES cells and mice

7.1. Introduction

The data revealed in this study has led to the hypothesis that the differential regulation of Pitx3 expression early in mDA development may determine whether mDA neurons will contribute to the SNc or VTA. Two main lines of evidence have contributed to this hypothesis, firstly, the analysis of *Pitx3* heterozygous mice led to the identification of two subgroups of mDA neurons at an early stage of mDA neuron generation (E12.5). These two subgroups are identified by their differential temporal and topographical expression patterns of Pitx3 and TH. At E12.5 there is a subgroup present in a more ventral and lateral position that expresses Pitx3 but not TH and a subgroup present in a more dorsal and medial position that expresses TH but not Pitx3 (Ch. 4.2). Secondly, even though Pitx3 is expressed in both the SNc and VTA, the loss of Pitx3 results in the loss of TH expression in mDA neurons of the SNc and a loss of SNc mDA neurons via apoptosis (Ch's. 5 and 6), whereas the VTA remains relatively unaffected. As Pitx3 has different functions in the SNc and the VTA it is likely that its expression with respect to TH expression is regulated differentially in the SNc and VTA.

At E12.5 the architecture in the midbrain does not correlate with the organisation of the mDA neurons in the adult, hence it is not possible to ascertain which neurons will form the SNc and which will form the VTA from their anatomy. Additionally, no markers are known that reliably distinguish between SNc and VTA cells at this stage of development. However, the position of the ontogentically distinct mDA subgroups at E12.5 is indicative that the more lateral Pitx3^{+}TH^{-} group will migrate most laterally to form the SNc, whereas the Pitx3^{-}TH^{+} group are in a more medial start position so they will form the VTA (Ch. 4.2). The route by which mDA neuron progenitors migrate from the ventricular zone and come to their final destination is controversial. The so called 'inverted fountain' model proposes that mDA precursors are generated in the medial part of the neuroepithelium and migrate ventrally down the midline before migrating laterally to their final position (Hanaway et al., 1971; Kawano et al., 1995; Marchand and Poirier, 1983). On the other hand, it has been proposed that mDA precursors generated from the medial ventral one-third of the ventricular zone and

migrate radially, perpendicular to the ventricle (Smidt et al., 2004) (see Ch 8.5 and Fig. 8.1). The orientation of the TH^+ neurons in the midbrain at E12.5 in this study supports this 'inverted fountain' model of mDA neurogenesis. Analysis of mDA cell bipolar orientation at E12.5 revealed that the TH^+ cells located closer to the medial part of the neuroepithelium exhibited an orientation suggestive of a vertical migration route, whereas TH^+ cells located in a lateral position had an orientation suggestive of a horizontal migration route (Ch 4.2.3). Taking these data together it seems probable that the two ontogenetically distinct subpopulations observed at E12.5 correspond with SNc and VTA forming cell populations and that the mDA cells that express Pitx3 before TH will form the SNc, while the mDA cells that express TH before Pitx3 will form the VTA. Therefore, to test this hypothesis, cell fate tracking experiments are required to track the fate of cells from the E12.5 $Pitx3^+TH^-$ population to their final destination.

7.2. Cell fate tracking strategy

A number of methods both genetic and non-genetic can be used for tracking cell lineage. The non-genetic approaches involve labelling cells based on their location with a marker such as a dye or a replication incompetent retrovirus. The vital carbocyanine dyes, such as DiI and DiO, are lipid-soluble and water-insoluble so they partition to the cell membrane and do not transfer between cells. In order to track cell fate these dyes can be injected into the tissue of interest, however it is difficult to apply them to a single cell and they are usually used to label groups of cells (Stern and Fraser, 2001). For example, DiI has been injected into the developing mouse hindbrain in combination with in vitro whole embryo culture to map the patterns of cranial neural crest cell migration into the developing branchial arches (Trainor et al., 2002). As the Pitx3- and TH-expressing cell populations are in such close proximity to each other at E12.5 it would be technically unachievable to label either population exclusively using this method. Furthermore, it is not feasible to culture an embryo of this age for the required amount of time. Another way to non-genetically label cells is to introduce a replication incompetent retroviral vector encoding a reporter gene. The replication incompetent retroviral vector is able to infect cells and transduce a non-viral gene, it is passed on to all daughter cells but it does not infect neighbouring cells (Cepko et al., 1998). Such a low concentration of the virus can be given that single cells are labelled, however infection is random and cells cannot be selected. In addition only mitotic cells can serve as successful hosts

for retroviruses (Cepko et al., 1998). Thus, as well as being unable to label specific cells this method is also unsuitable to label Pitx3-expressing cells as they are post-mitotic (Ch. 4.2.2).

To label and track the progeny of single cells a mutated form of the reporter gene *lac*Z called *laac*Z has been developed. *Laac*Z has an internal duplication that results in the premature termination of translation and so does not produce *lac*Z enzyme. However, a rare (<1 in 10000) intragenic homologous recombination event regenerates the functional *lac*Z gene (Bonnerot and Nicolas, 1993). This technology can be used for retrospective clonal analysis in a specific cell or tissue type by placing the *laac*Z gene under the control of a specific promoter. This results in the random labelling of relatively few cells and all of their progeny during development (Mathis et al., 1997). Although, again this method is not suitable to track the fate of the SNc or VTA cells as it is a tool for clonal analysis and the Pitx3-expressing midbrain cells are post-mitotic.

Another approach is to take advantage of endogenous gene expression patterns to label specific cell lineages. The simplest transgenic method involves placing the expression of a reporter gene under the control of the gene of interest, as in the case of the *Pitx3*-GFP mice. However, this approach is unsuitable to test the lineage of SNc and VTA as they both express Pitx3 and there are no well characterised genes that are expressed exclusively in either cell population during development. The Cre/loxP system can also be used to label cells expressing a gene of interest. Two transgenic mouse lines are required, one containing a reporter gene that is disrupted by a loxP flanked sequence and under the control of a ubiquitous promoter and another that expresses Cre recombinase under the control of the promoter of the gene of interest. Thus the reporter gene is only expressed in cells in which Cre is active and so able to excise the loxP flanked disruptive sequence and allow reporter gene expression (Zinyk et al., 1998). The main advantage of this over the simple transgene expression is that cells are irreversibly marked by the expression of Cre, so progeny of the cells that no longer express the gene of interest will still express the reporter gene.

An enhancement of the Cre/LoxP system that includes temporal control uses the tamoxifen-inducible form of Cre recombinase, $CreER^{T2}$, in which Cre recombinase has been fused to a mutated version of the estrogen receptor (ER) ligand binding

domain. CreER^{T2} is activated by the artificial ligand tamoxifen but not by estradiol that may be present endogenously in mice (Feil et al., 1996; Feil et al., 1997). This system is ideal to track the migration and final destination of the earliest Pitx3-expressing midbrain cells as it allows control of the reporter allele spatially by restricting it to Pitx3-expressing cells and temporally by administering tamoxifen to activate Cre and consequently the reporter expression.

The feasibility of this approach is supported by recent studies that have employed a similar strategy to fate map other neuronal lineages during embryonic development. For example, the pattern of cell migration in the cerebellum and midbrain-hindbrain regions have been analysed using En1 and En2-CreER^{T2} (Sgaier et al., 2005) and Wnt1, En1 and Gli1-CreER^{T2} (Zervas et al., 2004) mouse lines, respectively. Tamoxifen was administered at various stages between E8.5 and E11.5 and this resulted in labelling of cells expressing CreER^{T2} 24 to 36 hours after tamoxifen administration via oral gavage (Sgaier et al., 2005; Zervas et al., 2004). Furthermore, it has been noted that nuclear translocation of CreER occurs within 6 hours, is sustained for approximately 24 hours and no further recombination events take place 48 hours after tamoxifen administration (Hayashi and McMahon, 2002; Zervas et al., 2004). Therefore, to track the fate of the E12.5 Pitx3^{+}TH^{-} cells it is estimated that tamoxifen would be given at around E10.5 in order that Cre will only be active until E12.5 (Fig. 7.1). Although, the timing and dose of tamoxifen will need to be optimised for this particular experiment to ensure that only cells expressing Pitx3 at E12.5 are labelled.

In addition to the use of the Pitx3-CreER^{T2} mice for tracking the fate of the early Pitx3-expressing cells, they should be a useful tool for the inactivation of specific genes in mDA neurons in a temporally controlled manner. A number of mouse lines containing floxed genes have already been created and a database of these lines is available (http://www.mshri.on.ca/nagy/floxed.html). Pitx3-CreER^{T2} mice will be particularly useful for investigating the specific mDA role of genes that are more broadly expressed or have lethal knock-out phenotypes. In addition the temporal regulation provided by tamoxifen administration could be employed to create a more accurate animal model of Parkinson's disease by disrupting certain genes at later stages of adulthood (Ch. 8.7).

7.3. Results

7.3.1. Construction of Pitx3-CreER^{T2} targeting vector

In order to have the CreER^{T2} under the control of the endogenous Pitx3 promoter a promoterless En2SA-CreER^{T2} cassette and a pCMV*HygroTK* selection cassette were used. The Engrailed 2 splice acceptor (En2SA) allows the cassette to be inserted into an intron with efficient mRNA processing (Gossler et al., 1989). The *HygroTK* is a result of fusing the *hygromycin phosphotransferase* (*Hygro*) gene with the herpes simplex virus type-1 *thymidine kinase* (*TK*) gene, which confers hygromycin B resistance for positive selection and ganciclovir sensitivity for negative selection (Lupton et al., 1991). Additionally, the *HygroTK* was flanked by loxP sites (floxed) so that it could be used to screen for homologous recombinant ES cell clones and then later removed using Cre recombinase. An existing vector (Pitx3KO3, created by S. Zhao) containing a pCMV*HygroTK* gene flanked by a 1.9kb *Pitx3* 5' homologous arm and a 5.8kb *Pitx3* 3' homologous arm was used as the basis for the CreER^{T2} targeting vector. The *HygroTK* is under the control of the cytomegalovirus immediate early-enhancer (pCMV) which gives high levels of transgene expression in ES cells (Ward and Stern, 2002). In order to get the En2SA and CreER^{T2} fragments in sequence together and flanked by suitable restriction sites, a 2.1kb NotI-KpnI CreER^{T2} fragment from a pBluescript vector containing CreER^{T2} (pBSCreER^{T2}, a gift from L. Grotewold) and a 1.9kb XhoI-NotI En2SA fragment were three-way cloned into a XhoI-KpnI cut pSP72poly4 vector to create pSP72poly4En2SA-CreER^{T2}. The pSP72poly4En2SA-CreER^{T2} vector was cut with SalI to release the 3.9kb En2SA-CreER^{T2} fragment which was inserted into the XhoI sites of Pitx3KO3 in between the *Pitx3* 5' homologous arm and the floxed pCMV*HygroTK*, to create *Pitx3*-CreER^{T2} plasmid (Fig. 7.2). This strategy is similar to the *Pitx3*-GFP targeting strategy and results in the replacement of the entire *Pitx3* coding sequence. As the mice used in the fate mapping study will be heterozygous for the mutated *Pitx3* allele, the development and survival of mDA neurons will not be affected.

7.3.2. Electroporation and screening for homologous recombinant ES cells

The targeting vector was electroporated into E14TG2a ES cells as described in materials and methods (Ch. 2.5). Following electroporation the selection of ES cell transfectants was achieved by adding hygromycin B to the media. 583 hygromycin B resistant clones were picked and expanded in 24-well plates before splitting to freeze

half and use half for genomic DNA preparation. In order to screen for homologous recombinant ES cell clones, genomic DNA from all of the hygromycin-resistant clones was digested with HindIII enzyme and Southern blotted. The probe used for Southern blotting was a 3kb fragment corresponding to the endogenous *Pitx3* 5' region immediately upstream of the targeted *Pitx3* 5' homologous arm. This probe detects the 9.5kb fragment of the wild type allele and the 8.0kb fragment of the mutant allele (Fig 7.3). Three of the 583 ES cell clones screened displayed the 9.5kb fragment for the wild type allele plus the 8.0kb fragment for the recombinant allele. The rest of the clones displayed only the 9.5kb fragment for the wild type (Fig. 7.3). Thus the targeting efficiency for this experiment was 0.5%. The positive clones which had been frozen at -80°C were recovered and thawed (Ch. 2.4), however, only one of the three positive clones, clone 450, survived the freeze-thaw process. This clone was used for all further experiments.

7.3.4. Excision of selection cassette

Male transgenic mice carrying the *TK* gene rarely transmit the transgene and are usually infertile as a result of ectopic reporter expression in the testis (Al-Shawi et al., 1988; al-Shawi et al., 1991; Braun et al., 1990; Ellison et al., 2000). Therefore, to allow transmission of the CreER^{T2} transgene the pCMV*HygroTK* selection cassette was removed from the targeted clone 450 line of ES cells. Cre recombinase was transiently expressed in 450 ES cells using the plasmid pCAGCre in which Cre is under the transcriptional control of the CAG promoter (which possess the CMV immediate early enhancer and chicken β-actin promoter). Ganciclovir was used to select for ES cells lacking the *TK* gene and 12 ganciclovir resistant clones were picked (Ch 2.5).

7.3.5. Chimera production and germline transmission

Four of the pCMV*HygroTK*-deleted subclones were selected to make chimeras for germline transmission of the *Pitx3*-CreER^{T2} allele (Table 7.1). Chimeras were made using the diploid aggregation method (Ch.2.4.5) performed by G. Russel, K. Jones, J. Ure and J. Agnew. Chimeric pups were identified by coat colour chimerism. The E14TG2a ES cells are from Ola/129 mice which carry the agouti gene at the Aw locus and chinchilla at the C locus resulting in light buff coat colour and the host embryos were C57Bl/6 X CBA F1 mice which are agouti (brown coat colour). Of a total of 191 blastocysts implanted and 26 pups born, 4 of these were chimeric. The

four male chimeric mice were mated with C57Bl/6 (black coat) female mice and one of the clone 450-8 chimeric mice was germline transmitting and produced *Pitx3*-CreER^{T2} heterozygous offspring (Fig.7.4).

Table 7.1. Summary of chimera production and germline transmission

ES cell clone	Blastocysts implanted	Pups born	Chimeras (male)	Germline chimeras
450-1	58	2	1 (1)	-
450-2	53	7	1 (1)	-
450-6	38	4	0	-
450-8	42	13	2 (2)	1

7.4. Summary

Mice containing CreER^{T2} under the control of the endogenous Pitx3 promoter were created in order to perform a cell fate tracking study to test the hypothesis that the mDA cells that express Pitx3 before TH will form the SNc. A targeting construct was made using an En2 splice acceptor-CreER^{T2} cassette inserted in between 5' and 3' Pitx3 homologous arms. This targeting vector was electroporated into ES cells and incorporation of the CreER^{T2} cassette into the ES cell DNA via homologous recombination occurred at a rate of 0.5%. One of the CreER^{T2} containing clones was used to create germline transmitting chimeric mice.

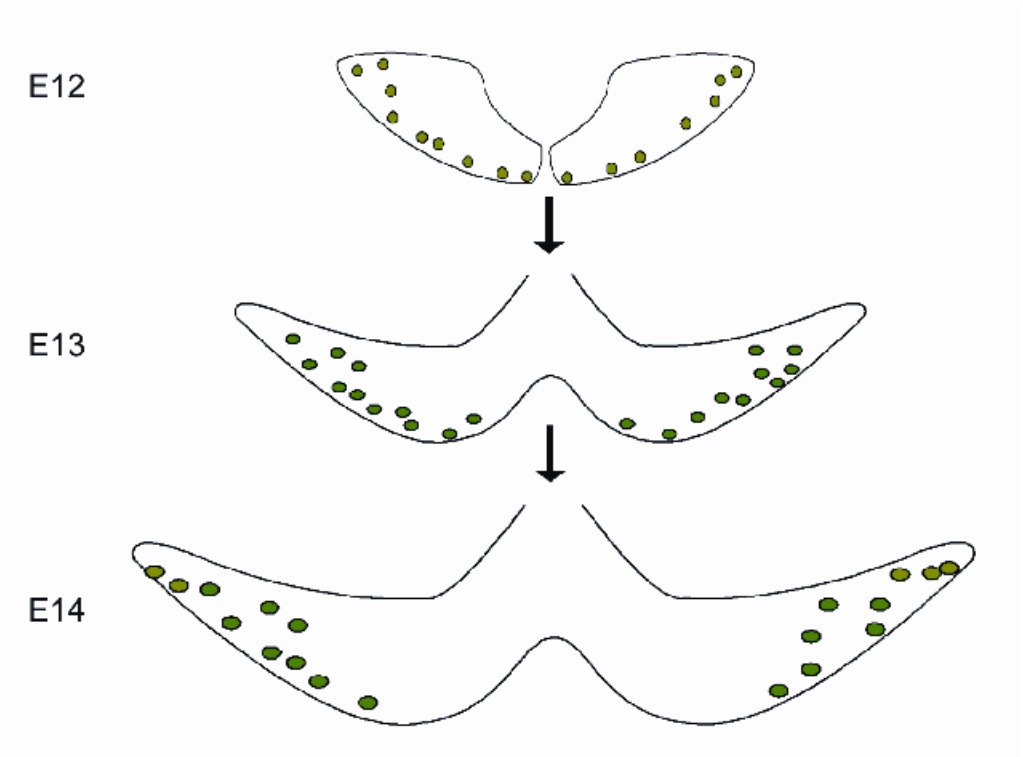

Figure 7.1. Diagram of cell fate tracking strategy

Tamoxifen will be administered to mother at E10-E11 (exact time will have to be determined empirically) in order to label only Pitx3-expressing cells at E12.5 and no later. Cre will be translocated to the nucleus in the Pitx3-CreERT2 expressing cells at E12.5 and will act to recombine the loxP sites which will remove the “stop” codon therefore activating the GFP expression. These cells labelled at E12.5 will remain GFP-labelled throughout development. Brains can then be collected at later stages of embryonic development or post-natal and the location of the GFP-labelled cells can be analysed.

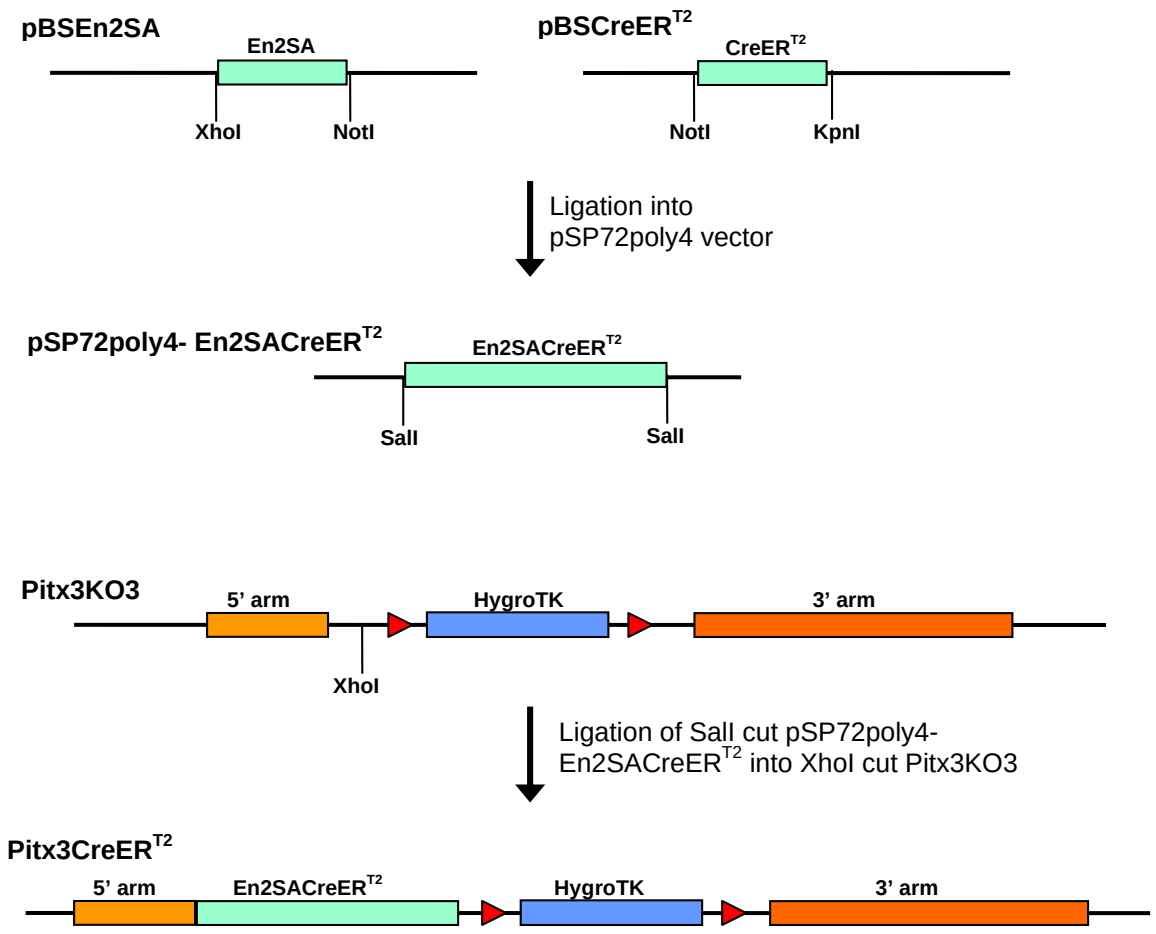

Figure 7.2. Targeting vector construction

A 1.9kb XhoI-NotI En2SA fragment and a 2.1kb NotI-KpnI CreER^{T2} fragment from pBSCreER^{T2} were three-way cloned into a pSP72poly4 vector cut with XhoI-KpnI to create pSP72poly4En2SA-CreER^{T2}. Subsequently, the pSP72poly4En2SA-CreER^{T2} plasmid was cut with SalI to release the 3.9kb En2SA-CreER^{T2} fragment which was ligated into the Pitx3KO3 that had been cut with XhoI, inserting En2SA-CreER^{T2} in between the *Pitx3* 5' homologous arm and the floxed pCMV*HygroTK* to create the *Pitx3*-CreER^{T2} plasmid. See appendix for plasmid maps.

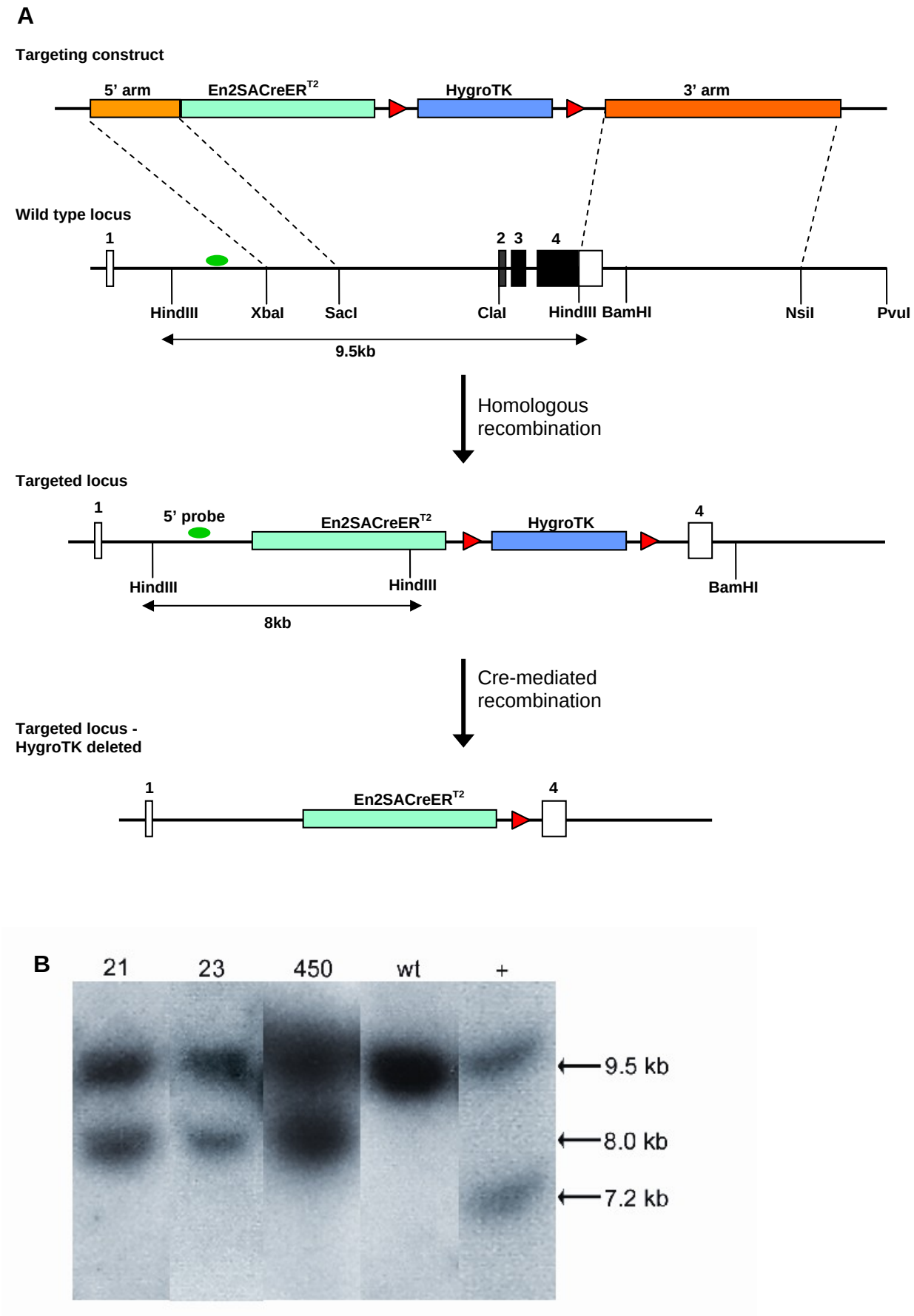

Figure 7.3. Southern blotting screening strategy for correctly targeted clones

(A) From the top is the *Pitx3*-CreER^{T2} targeting construct, the wild type *Pitx3* locus and the targeted *Pitx3* locus. The boxes on the *Pitx3* locus represent the *Pitx3* exons 1 to 4, blank boxes are non-coding and filled boxes are coding sequence. Homologous recombination at the *Pitx3* 5' and 3' homologous arms results in the replacement of the entire coding sequence of Pitx3 with the En2SACreER^{T2}-*HygroTK* cassette. Selection of clones in which homologous recombination had occurred was achieved by Southern blotting genomic DNA digested with HindIII. The location of the 3kb 5' probe is indicated by a green oval, thus it detects the 9.5kb fragment of the wild type allele and the 8.0kb fragment of the mutant allele.

(B) Southern blot showing the three positive clones 21, 23 and 450, an example of wild type clone (wt) and positive control DNA (+) from the *Pitx3*-GFP ES cells (Ch. 3.1). The wild type band is at 9.5kb, the *Pitx3*-CreER^{T2} band at 8.0kb and the *Pitx3*-GFP band at 7.2 kb.

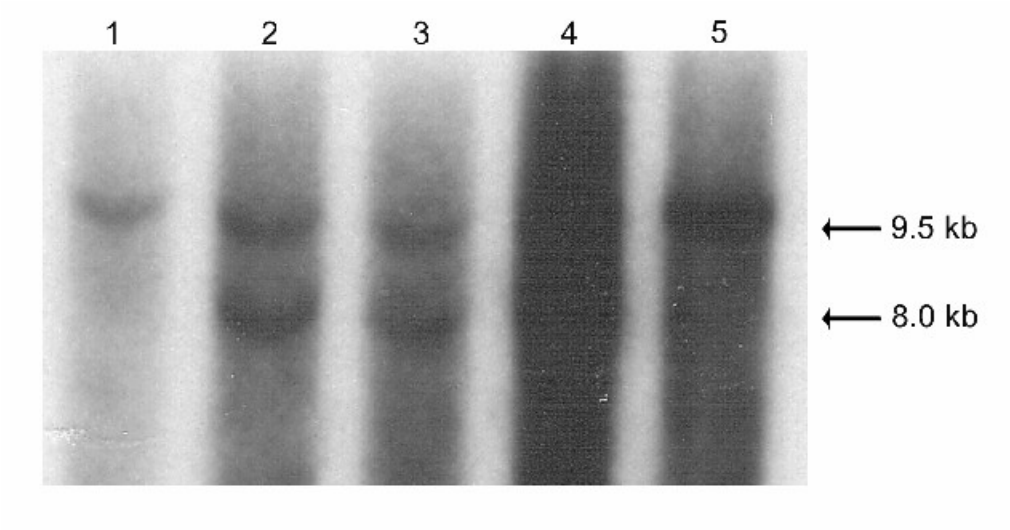

Figure 7.4. Southern blotting for detection of germline transmission

Southern blot showing genomic tail DNA from three pups (lanes 1 to 3) from a mating with chimeric mouse 450-8 (strategy as in Fig. 7.3). Two of the pups are heterozygous for the *Pitx3*-CreER^{T2} allele (8.0kb and 9.5kb band, lanes 2 and 3) and one is wild type (9.5kb band only, lane 1). Lane 4 shows positive control DNA from clone 450 ES cells (8.0kb and 9.5kb band) and lane 5 is negative control DNA from wt mouse tail (9.5kb band only).

Chapter 8

Discussion

The mDA neurons have a variety of key functions in the brain, the SNc mDA neurons in motor control and the VTA mDA neurons in mood and reward pathways. Degeneration of the SNc neurons results in PD, whereas the dysfunction and/or degeneration of VTA neurons is implicated in addiction and schizophrenia. Therefore, there is much interest in the study of mDA neuron physiology, development and pathogenesis. In order to understand more about the physiology and pathogenesis of these neurons and to advance the derivation of mDA neurons from ES cells, knowledge of the regulation of their development is required. So far, a small number of factors known to be involved in the development and survival of mDA neurons have been identified including Nurr1, Engrailed 1 and 2 and Lmx1b. However, the signalling cascades and interactions between the factors involved remain to be uncovered. In this thesis I have demonstrated the importance of one other factor, the homeodomain transcription factor Pitx3. Pitx3 is required for TH expression and cell survival specifically in SNc mDA neurons, although it is expressed in both the SNc and VTA mDA neuron populations. Furthermore, this study has shown that at around the start of mDA neuron generation there are two previously unrecognised ontogenetically distinct subsets of mDA neurons based on their expression of Pitx3 and TH. Currently there is little known about the differences between SNc and VTA mDA neuron development, however, these findings may provide the basis for diverse developmental pathways of the anatomically and functionally distinct SNc and VTA subpopulations.

8.1. *Pitx3*-GFP is an accurate marker of Pitx3 expression

Previously a *Pitx3*-GFP reporter mouse line was created to act as a reporter of *Pitx3* gene expression and to create a mutation in the *Pitx3* gene which resulted in a *Pitx3* null allele. The first aim of this thesis was to validate the *Pitx3*-GFP reporter mouse line as a tool for studying Pitx3 expression. It was found that the expression of the *Pitx3*-GFP reporter in phenotypically normal *Pitx3*-heterozygous embryos mirrored that of Pitx3. *Pitx3*-GFP was detected in the midbrain, lens, somites, cranial facial muscles and the tongue, which correlates with those sites previously reported for

Pitx3 mRNA expression (Semina et al., 2000; Semina et al., 1997; Smidt et al., 1997). So far, the requirement for the function of Pitx3 in the eye and the midbrain has been established (Rieger et al., 2001; Semina et al., 2000), however, no data is available on any role of Pitx3 in the other sites of expression including the tongue, head mesenchyme and somites.

In the midbrain, the first stage at which *Pitx3*-GFP and Pitx3 protein expression could be detected was at E12.5, yet *Pitx3* mRNA expression has been reported to be first detected in the midbrain at E11.5 (Smidt et al., 1997). As this study was looking at protein rather than mRNA expression this delay may be due to the time it takes for *Pitx3*-GFP mRNA to be translated and folded into protein. Also, our technique may not be as sensitive as the radioactive in situ technique used to reveal Pitx3 mRNA expression at E11.5 (Smidt et al., 1997). Using an antibody against Pitx3 revealed that *Pitx3*-GFP expression exactly represented Pitx3 protein expression in the midbrain at E12.5. This demonstrates that *Pitx3*-GFP is a faithful marker of Pitx3 protein expression.

A recent study had reported that Pitx3 was expressed in only some cells of the adult SNc and VTA. It claimed that in the SNc Pitx3 was expressed in the ventral mDA neurons but not in the dorsal mDA neurons, and that throughout the VTA Pitx3 was only expressed in around half of the mDA neurons (Van Den Munckhof et al., 2003). In contrast, my work has demonstrated that in the adult midbrain Pitx3 is expressed in almost all TH-expressing cells in the SNc and VTA and vice versa (Ch. 3.2). Another recent study has also shown the co-expression of Pitx3 and TH in all adult mDA neurons (Smidt et al., 2004), which adds further support for Pitx3 as a marker for mDA neurons of the adult SNc and VTA.

8.2. Pitx3 is required for the regulation of TH expression in SNc mDA neurons

A role for Pitx3 in *TH* regulation has previously been suggested by in vitro promoter studies. These studies have shown that there is a response element for Pitx3 in the *TH* promoter which Pitx3 is able to bind to and confer a transcriptional effect in vitro (Cazorla et al., 2000; Lebel et al., 2001). This thesis however, provides the first in vivo evidence of a role for Pitx3 in *TH* regulation and reveals that this function of Pitx3 is restricted to a subset of mDA neurons. Firstly, based on the finding that there

are around 50% fewer *Pitx3*-GFP^{+}TH^{+} cells but no change in the number of *Pitx3*-GFP^{+}TH^{-} cells at E12.5, this study demonstrates that Pitx3 is required for the expression of TH in some Pitx3-expressing cells and thus for the transition from post-mitotic mDA progenitor cells (*Pitx3*-GFP^{+}TH^{-}) to mDA neurons (*Pitx3*-GFP^{+}TH^{+}) at E12.5. At E14.5 it was shown that the specific loss of TH expression was restricted to *Pitx3*-GFP^{+} cells of the SNc whereas the VTA cells mostly co-expressed *Pitx3*-GFP and TH. Therefore the crucial role of Pitx3 in regulation of TH expression is restricted to SNc cells and is not in VTA cells. The in vitro promoter studies on Pitx3 regulation of the *TH* promoter have shown that the magnitude of the transcriptional effect of Pitx3 on the *TH* promoter was cell type-dependent (Cazorla et al., 2000; Lebel et al., 2001). Although none of the cell lines used in these studies were mDA neuron-derived, the results imply that a similar cell type-dependent mechanism could take place between mDA neuron subtypes. Pitx3 may act on its own to activate the *TH* promoter or it may interact with other molecules to achieve this effect. One candidate Pitx3 interacting partner that is expressed in all mDA neurons is Nurr1, which functions as a general *TH* regulator as TH expression is lost in both the SNc and VTA of *Nurr1* mutant mice (Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). One study has found that Nurr1 can act co-operatively with Pitx3 to enhance the effect of Pitx3 on the *TH* promoter (Cazorla et al., 2000), however another study has refuted this claim reporting that Nurr1 does not enhance the ability of Pitx3 to activate the *TH* promoter (Lebel et al., 2001). These studies were performed using different cell systems, Neuro2A and P19 cells respectively, so this discrepancy between the studies may imply that the combined effects of Pitx3 and Nurr1 may be cell type-dependent. Furthermore, the activation of TH gene expression by Nurr1 has been reported in the context of cultures of adult rat hippocampal progenitor cells (Sakurada et al., 1999), SK-N-BE(2)C and HeLa cells (Kim et al., 2003). However, in a number of cell types it was shown that Nurr1 had little or no effect on TH expression suggesting that the transactivation of the *TH* promoter by Nurr1 is also cell type-specific (Kim et al., 2003).

8.3. Pitx3 is required for the survival of SNc mDA neurons

This study has demonstrated that there is a progressive loss of SNc mDA neurons in *Pitx3* null mice starting at an early stage of mDA generation to result in a complete absence of SNc mDA neurons in adult *Pitx3* null mice. At E12.5, the first stage at which Pitx3 protein can be detected, there is a 50% loss of *Pitx3*-GFP^{+}TH^{+} cells. By

E14.5 there is around a 60% loss of *Pitx3*-GFP^{+}TH^{+} cells in the SNc of *Pitx3* null mice. Eventually, by adulthood there is a complete loss of mDA neurons in the SNc of *Pitx3* null mice. This supports the concept that Pitx3 is dispensable for the specification of post-mitotic mDA progenitor neurons, rather it is required for the survival and maintenance of a population of post-mitotic mDA (i.e.TH^{+}) neurons.

An intriguing finding at E12.5 was the significant loss of *Pitx3*-GFP^{-}TH^{+} cells in *Pitx3* null mice at E12.5. As these cells are not yet expressing Pitx3 it is surprising that they are affected in the *Pitx3* null mice. One explanation for this could be that these cells have just begun to express Pitx3 and the expression level is not yet detectable by immunostaining. On the other hand these cells may not be expressing Pitx3, but may require factors from the *Pitx3*-GFP^{+} cell population for their induction and/or maintenance. This hypothesis could be addressed by chimera experiments with *Pitx3* null ES cells and wild type embryos or vice versa. For example, it would be expected that the loss of *Pitx3* null *Pitx3*-GFP^{-}TH^{+} cells at E12.5 would be rescued if there was a high contribution of wild type cells compared to *Pitx3* null cells in the midbrain. Similarly you would expect loss of wild type Pitx3^{-}TH^{+} cells at E12.5 if there was a high contribution of *Pitx3* null cells and a low contribution of wild type cells in the chimeric midbrain.

From the TUNEL assay, Nissl staining and primary culture results it is likely that the absence of any *Pitx3*-GFP+ and TH+ cells in the *Pitx3* null adult SNc is due to apoptotic cell death during development. The TUNEL assay showed that around double the number of *Pitx3*-GFP^{+} cells in the *Pitx3* null SNc are undergoing apoptosis compared to the Pitx3 heterozgyous SNc and VTA or the *Pitx3* null VTA. The primary culture of E14.5 midbrain revealed comparable results to the TUNEL assay with around a two-fold increase in the number of *Pitx3*-GFP^{+} cells in the *Pitx3* null culture undergoing apoptosis as compared to the *Pitx3* heterozygous culture. As the *Pitx3* null SNc cells lose or fail to express TH it was possible that these cells did not die but changed fate into another neuronal subtype. As well as dopaminergic neurons, GABAergic neurons are also present in the VTA and SNc. The adult VTA and SNc contain around 15-25% GABAergic neurons whilst the remainder are dopaminergic (Bayer and Pickel, 1991). The results show that none of the E12.5 or E14.5 *Pitx3*-GFP+ neurons are GABAergic and that approximately all *Pitx3*-GFP+ cells expressed a DA marker (En1). However, the possibility of fate change in *Pitx3* null SNc cells cannot be ruled out, as if the cells no longer express *Pitx3*-GFP, their fate cannot be traced.

Taking these results together this study has revealed that the homeodomain transcription factor Pitx3 is required for TH expression and survival of SNc mDA neurons but not VTA neurons. A loss or downregulation of TH in mDA cells does not impair their survival, in *TH* knock-out mice the mDA neurons were found to be intact as revealed by staining with an antibody against L-aromatic amino acid decarboxylase (AADC), which is the enzyme that converts L-DOPA to dopamine (Zhou and Palmiter, 1995). This suggests that the two functions of Pitx3 in TH regulation and survival in SNc mDA neurons are likely to be regulated separately.

As the SNc neurons appear to die via apoptosis in *Pitx3* null mice, Pitx3 may be involved in the regulation of pro- or anti-apoptotic genes. Neuronal apoptotic cell death is a well recognised phenomenon characterised by morphological cell changes including chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage and plasma membrane blebbing (Yuan et al., 2003). More recently the molecules and cellular processes regulating apoptotic cell death are being uncovered. So far, some key molecules that are implicated in neuronal apoptosis are members of the Bcl-2, caspase and Apaf-1 families. The Bcl-2 family has both pro- and anti-apoptotic members, for example, Bcl-2 and Bcl-x_L are anti-apoptotic Bcl-2 proteins and Bax and Bak are pro-apototic Bcl-2 proteins that are expressed in the embryonic nervous system (Krajewska et al., 2002). The exact mechanisms by which Bcl-2 proteins act remain unclear, however it has been shown that mitochondrial release of cytochrome *c* is a crucial event in caspase activation and that Bcl-2 family members can regulate outer mitochondrial membrane permeability and thus release of cytochrome *c* (Harris and Thompson, 2000). Bcl-x_L is a member of the Bcl-2 family that has been linked with apoptosis in SNc mDA neurons (Savitt et al., 2005). Bcl-x_L is a product of the *Bcl-x* gene which can be alternatively spliced to produce two protein isoforms, the anti-apoptotic Bcl-x_L, which mediates most nervous system effects, and the pro-apoptotic Bcl-x_S, which is expressed at low or undetectable levels in the brain (Krajewska et al., 2002). Mutation of the *Bcl-x* gene in mice results in a huge loss of neurons and haematopoietic cells and embryonic lethality at E13.5 in *Bcl-x* null mice (Motoyama et al., 1995). The effects of *Bcl-x* proteins in dopaminergic neurons were investigated by performing a conditional *Bcl-x* knock-out in which mice with Cre expressed under the control of the *TH* promoter were crossed with mice containing a loxP flanked *Bcl-x* gene. In the SNc of these mice there were around a third fewer mDA neurons and a similar loss of neurons was observed in all catecholaminergic cells studied. Many of the remaining neurons expressed TH and Bcl-x, suggesting

that the Cre-mediated excision had not worked in these cells (Savitt et al., 2005). Thus, it is likely that in the majority of SNc mDA cells Bcl-x_L is required for cell survival. The expression profile of Bcl-x_L in the developing nervous system peaks at around E13 to P7 (Krajewska et al., 2002), which coincides with the appearance of mDA neurons and the death of SNc mDA cells in Pitx3 null mice (Ch. 5.2). Cell death in *Bcl-x* knock-out mice occurs in post-mitotic but immature neurons (Motoyama et al., 1995; Roth et al., 1996). Additionally there is an increase in Bcl-x_L expression during the differentiation of DA neurons from ES cells (Shim et al., 2004). This data implies a possible role for Pitx3 in regulation of the *Bcl-x* gene in the SNc, either directly or by a downstream target of Pitx3.

As Pitx3 is expressed in both the SNc and VTA mDA neurons it might be expected that it functions similarly in both populations. However analysis of the *Pitx3* null mice has shown that it is specifically required in the SNc mDA neurons where it has at least two functions, one is the regulation of TH expression and the other is cell survival (Ch. 5.2). Therefore, it is possible that there are cell-specific factors that are yet to be identified, which interact with Pitx3 to produce these cell-specific effects. Although no evidence exists for Pitx3 interacting partners in mDA neurons, it seems likely from studies on similar proteins. A number of transcriptional targets and interacting partners have been identified for Pitx1 and Pitx2. Both of these Pitx factors are able to act as transcriptional regulators of many pituitary hormones including POMC (Lamonerie et al., 1996), glycoprotein hormone α-subunit (αGSU), growth hormone (GH), prolactin (Szeto et al., 1996; Tremblay et al., 1998), thyroid stimulating hormone (TSH) (Tremblay et al., 1998), luteinizing hormone β (LHβ) (Quirk et al., 2001), follicle-stimulating hormone β (FSHβ) (Zakaria et al., 2002) and gonadotrophin-releasing hormone (GnRH) (Tremblay and Drouin, 1999). Pitx1 displays cooperative interactions with both early growth response 1 (EGR1) and steroidogenis factor 1 (SF1, NR5A1) (Tremblay and Drouin, 1999; Tremblay et al., 1999). While Pitx2 has been shown to interact with the POU homeodomain protein Pit-1 (Bach et al., 1997; Szeto et al., 1996). Other studies have established that Pitx homeodomain family members can activate transcription via direct protein-protein interactions with cell restricted transcription factors such as NeuroD1, a member of bHLH family of transcription factors, and Tpit, a T-box factor (Lamolet et al., 2001; Poulin et al., 2000). The interaction of Pitx factors with bHLH factors is interesting as bHLH factors are involved in neurogenesis and promote a neural fate in vivo (Lee, 1997). For example, in sympathetic neurons the *paired* like transcription factors

Phox2a and Phox2b interact with bHLH factors to regulate neurotransmitter phenotype by activating dopamine β-hydoxylase (Morin et al., 1997; Pattyn et al., 1997). Another potential Pitx3 interacting partner is Ldb1 which is a cofactor that is able to bridge between LIM-HD proteins and Pitx proteins (Bach et al., 1997). The expression of Lmx1b not only overlaps with Pitx3 in mDA neurons, but also with Pitx2 in the subthalamic nucleus (STN), posterior hypothalamus and some mammillary nuclei where Ldb1 is also expressed (Asbreuk et al., 2002). Therefore, Ldb1 may mediate cooperation between Lmx1b and Pitx factors in these regions.

8.4. Pitx3 acts in a cell autonomous manner

Studies have shown that the homeodomain transcription factor En2 can be secreted and taken up by other cells in vitro, suggesting that it may have the capacity to act as an intercellular signal in vivo (Joliot et al., 1998; Maizel et al., 1999). A chimera study was carried out to investigate the possibility that Pitx3 may be able to act intercellularly, or if any molecules downstream of Pitx3 were present in the midbrain environment and able to rescue *Pitx3* null SNc cells. The chimera study indicates that *Pitx3* null ES cells are severely compromised in their ability to contribute to the SNc compared to *Pitx3* heterozygous ES cells. This suggests that neither Pitx3 nor any of its downstream targets present in the wild type embryo are able to act in a cell non-autonomous manner to enable the *Pitx3* null cells to integrate and survive in the SNc. Therefore, it appears that Pitx3 acts in a cell autonomous manner in mDA neurons. Due to the competition between wild type and mutant cells in a chimera situation, chimera studies are able to give additional insights into the phenotype of mutations. This chimera study revealed that the Pitx3 heterozygous ES cells were also compromised in their ability to contribute to the SNc. As the *Pitx3* heterozygous mDA phenotype cannot be distinguished from wild-type this *Pitx3* haploinsufficiency is only evident when these cells are challenged as in the chimera situation where there is competition between *Pitx3* heterozygous and wild type cells. Homeodomain transcription factor haploinsufficiency is common and especially manifests in many cases of human disease (Seidman and Seidman, 2002). For example, the loss of one allele of *Pitx2* causes Reiger Syndrome in humans and a similar phenotype in mice characterised by abnormalities of the anterior chamber of the eye, dental hypoplasia, craniofacial dysmorphism and iris hypoplasia, (Gage et al., 1999; Semina et al., 1997). Furthermore, cateracts and anterior segment mesenchymal dysgenesis (ASMD) in humans are caused by *Pitx3* haploinsufficiency (Semina et al., 1998). It

would be interesting to further investigate the haploinsuffiency of Pitx3 in the mDA neurons. This could be done by challenging the *Pitx3* heterozygous mDA neurons with mDA-specific toxic agents such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA), for example. This may reveal that *Pitx3* heterozygous mDA neurons are more vulnerable than wild-type neurons when challenged with toxins.

8.5. Pitx3 expression and function defines the SNc and VTA subgroups

The SNc and VTA mDA neurons are anatomically and functionally distinct groups of neurons, however, currently there are few markers that are able to distinguish between them. The most utilized markers are Girk2 and calbindin which are expressed in the SNc and VTA respectively, although this expression is not completely exclusive. The G-protein-gated inwardly rectifying K^+ channel Girk2 is expressed in most SNc mDA neurons and has been used as a marker for SNc neurons (Thompson et al., 2005), however, it is also expressed in the more lateral VTA mDA neurons (Schein et al., 1998). The calcium binding protein calbindin is expressed in the vast majority of the VTA mDA neurons, but many cells of the SNc have been reported to express calbindin as well (Liang et al., 1996). Furthermore, these markers can only distinguish between SNc and VTA neurons in adult mice and both Girk2 and calbindin are expressed in the SNc and VTA in embryonic and early postnatal mDA neurons (M. Parmar, unpublished observations). Therefore, in order to understand the differences between these neurons it is necessary to be aware of the different ontogeny and expression profiles of the SNc and VTA neurons.

In the adult mouse midbrain the vast majority of mDA neurons in the SNc and VTA express both TH and Pitx3. However, this study has revealed that Pitx3 has different roles in the SNc and VTA and that mDA neurons arise from two distinct subpopulations of cells identified by their differential expression profiles of Pitx3 and TH. At E12.5 a population of mDA neurons in the midbrain express TH but not Pitx3 and another population of cells express Pitx3 but not TH. It is likely that the $Pitx3^+TH^-$ population of cells at E12.5 are mDA precursors as they express the neuronal and mDA marker genes, βtubulin3 and En1, respectively, and they do not express markers for mitotic cells, glial cells or GABAergic neurons. Over the next few days of embryonic development the $Pitx3^-TH^+$ and $Pitx3^+TH^-$ cells begin to express Pitx3 and TH, respectively, and eventually almost all mDA neurons express both Pitx3 and TH.

The developmental heterogeneity within the mDA population revealed in this study implies that mDA neuron development proceeds via both Pitx3-dependent and Pitx3-independent mechanisms, and that this distinction in ontogeny is reflected in the selective vulnerability of the SNc mDA lineage in *Pitx3* null mice. It is not possible to identify which adult cell populations these E12.5 subgroups will form due to a combination of the architecture of the mDA neurons and the lack of markers able to distinguish between SNc and VTA neurons at this stage of development. However, there are a number of lines of evidence to suggest that these ontogenetically distinct subgroups at E12.5 do form the SNc and VTA, respectively.

Firstly, the position of these groups at E12.5 is indicative that the more lateral $Pitx3^{+}TH^{-}$ group will migrate most laterally to form the SNc, whereas the $Pitx3^{-}TH^{+}$ group are in a more medial start position so are more likely to form the VTA. The route by which mDA neurons migrate from the ventricular zone and come to their final destination is controversial. Some studies suggest that mDA precursors are generated in the most medial and ventral part of the neuroepithelium and migrate ventrally down the midline before migrating laterally to their final position, in a so called 'inverted fountain' model (Fig. 8.1.A) (Hanaway et al., 1971; Kawano et al., 1995; Marchand and Poirier, 1983). On the other hand, it has been proposed that mDA precursors are generated from the medial ventral one-third of the ventricular zone and migrate radially, perpendicular to the ventricle (Fig. 8.1.B) (Smidt et al., 2004). The orientation of the TH^{+} neurons in the midbrain at E12.5 in this study supports this 'inverted fountain' model of mDA neurogenesis. Analysis of mDA cell bipolar orientation at E12.5 revealed that the TH^{+} cells located closer to the medial part of the neuroepithelium exhibited an orientation suggestive of a vertical migration route, whereas TH^{+} cells located in a lateral position had an orientation suggestive of a horizontal migration route (Ch 4.2.3). Additional data in this study supporting the 'inverted fountain' migration pattern is that some *Pitx3*-GFP^{+} cells in a more medial position were βTubulin3-negative (Ch. 3.2). This would correlate with the 'inverted fountain' model of mDA neurogenesis and migration as the medial cells are less mature than the more lateral cells and thus have not yet started to express βTubulin3. If the 'inverted fountain' model of migration does occur then it is likely that the more ventral and lateral $Pitx3^{+}TH^{-}$ population at E12.5 will become the SNc while the more dorsal and medial $Pitx3^{-}TH^{+}$ population at E12.5 will become the VTA. However, the possibility that a combination of both modes of migration may occur cannot be ruled out.

Secondly, over the course of the next few days after the distinct subpopulations visible at E12.5, almost all mDA neurons in the midbrain of *Pitx3* heterozygous mice express both Pitx3 and TH except some at the most lateral region of the SNc at E14.5 which still are $Pitx3^{+}TH^{-}$ (Ch. 4.2). This further implies that the E12.5 $Pitx3^{+}TH^{-}$ population will become SNc neurons as the remnants of this population are present in the SNc primordium at E14.5. A third line of evidence is the fact that a loss of Pitx3 only affects the SNc neurons even though it is expressed in both SNc and VTA. This differential dependence on Pitx3 reveals that Pitx3 has diverse roles and may have distinct interacting partners in these functionally distinct mDA neuron populations. Thus, it is likely that there is a differential regulation of Pitx3 expression with respect to TH expression in the SNc and VTA.

8.5.1. Do the ontogenetically distinct subgroups at E12.5 distinguish the SNc and VTA?

This subregional heterogeneity may provide a basis for different developmental pathways for the anatomically and functionally distinct SNc and VTA subpopulations. To address this problem a fate mapping study has been designed to track the migration of the earliest Pitx3 expressing cells, i.e. the population of cells that are $Pitx3^{+}TH^{-}$ at E12.5. ES cells with $CreER^{T2}$ under the control of the endogenous *Pitx3* promoter have been generated and mice carrying this allele are in production (Ch. 7). By crossing these mice with another line of transgenic mice containing a Cre-dependent reporter gene under the control of a ubiquitous promoter, the expression of the reporter gene will be controlled both spatially and temporally. Thus the reporter gene expression will be dependent upon the expression of *Pitx3*-$CreER^{T2}$ and the presence of the synthetic ligand tamoxifen. Therefore, the cells that express Pitx3 at E12.5 can be labelled with the reporter then the midbrains can be examined at later stages of development to establish where the labelled cells are located. This will ascertain whether the E12.5 $Pitx3^{+}TH^{-}$ cell population contributes to the SNc, VTA or both areas and thus test the hypothesis discussed above. In addition this analysis should help to build up a clearer picture of the pattern of mDA neuron migration.

8.5.2. Further applications of *Pitx3*-$CreER^{T2}$ mice

As well as being a tool for tracking the fate of early Pitx3 expressing cells, the *Pitx3*-$CreER^{T2}$ mice may be used to conditionally knock-out genes in a mDA specific manner. This will be particularly useful to disrupt the expression of genes that may be involved in mDA neuron regulation but due to their other role(s) the conventional

knock-out is embryonic lethal or the disruption caused by the knock-out is too widespread in the CNS to distinguish the mDA phenotype. For example, Lmx1b is expressed in mDA neurons, however it is also expressed in other brain areas and throughout the mesencephalon and diencephalon from E7.5 (Smidt et al., 2000). Consequently, it is difficult to analyse the effect of a lack of Lmx1b on mDA neurons in the Lmx1b knock-out as there are large mid- and hindbrain deletions which may affect mDA neuron development (Smidt et al., 2000). Similarly in the Engrailed 1 and 2 double knock-out there is a substantial truncation of the midbrain and anterior hindbrain resulting in a loss of the isthmus and reduced levels of Shh and FGF8 (Simon et al., 2001). Therefore, a conditional knock-out of these genes using the *Pitx3*-CreER^{T2} mice may reveal the true function of these genes in mDA neurons.

In addition, the *Pitx3*-CreER^{T2} mice will be a useful tool to track the fate of *Pitx3* null SNc mDA neurons. The evidence in this study suggests that Pitx3 null SNc neurons die rather than change fate into another cell type (Ch. 6.2). However, the possibility that some of these cells down-regulate *Pitx3*-GFP and TH expression and begin to express other cell-type specific markers cannot be ruled out. The *Pitx3*-CreER^{T2} mice crossed with a Cre-dependent reporter mouse will be able to address this problem as all cells which express *Pitx3*-CreER^{T2} can be permanently labelled by Cre-mediated activation of the reporter gene expression. Thus, the fate of any labelled cells can be tracked.

8.6. Comparison of *Pitx3* null with *aphakia*

During the course of this work, a number of studies on the mDA phenotype of the naturally occurring mouse mutant *aphakia* were published. Of the studies that investigated the mDA phenotype during embryonic development, one reported no difference between *aphakia* and wild type mDA neurons at E12.5 (Van Den Munckhof et al., 2003). Another study showed that *aphakia* mice had aberrant positioning of TH$^+$ neurons at E12.5, with fewer neurons in a rostral-lateral position and more in a dorsal-medial position compared to wild type, although a reduction in TH$^+$ cell number was not detected (Smidt et al., 2004). These findings in *aphakia* mice are in contrast to the findings in this study of *Pitx3* null mice in which there is a loss of more than half of the TH$^+$ neurons at E12.5 compared to wild type (Ch. 5.2). Studies on aphakia also revealed a loss of the most lateral TH$^+$ cells at E14.5 (Nunes et al., 2003; Smidt et al., 2004), however no quantitative data is available to

determine if this loss is comparable to the *Pitx3* null phenotype in which there is a deficit in TH^+ neurons in the SNc of more than 50% at E14.5 (Ch. 5.3).

The majority of the studies on post-natal and adult aphakia mice were in concurrence with this study on *Pitx3* null mice, in that there is a complete loss of SNc neurons while the VTA remains much less affected (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004). On the contrary, van den Munckhof et al. report a loss of TH^+ neurons in the majority of the SNc except for the most dorsal tier of this structure and a loss of half the TH^+ neurons in the VTA. This correlates with their claims that Pitx3 is not expressed in most of the dorsal SNc mDA neurons and only expressed in around half of the VTA mDA neurons (Van Den Munckhof et al., 2003). However, these observations were neither supported by this study nor by that of Smidt et al., which clearly demonstrate that Pitx3 and TH expression overlap completely throughout the SN and VTA (Ch. 3.2) (Smidt et al., 2004) and that loss of Pitx3 results in a complete loss of SNc mDA neurons (Ch. 5.2).

The four published studies on the midbrain phenotype of *aphakia* mice support the findings in this study that *Pitx3* null SNc neurons die. Nissl staining in aphakia mice revealed an absence or a reduction in the number of neurons in the SNc of *aphakia* mice (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). Furthermore, evidence for apoptotic cell death was revealed by TUNEL labelling in the aphakia mice (Van Den Munckhof et al., 2003) which is comparable to the results in this study (Ch. 6.2).

The *aphakia* studies and this study of *Pitx3* null reveal that the phenotypes of these mice are largely similar. However, the apparently more severe mDA defect in the *Pitx3* null is probably due to the creation of a true null mutation by gene targeting, since the entire *Pitx3* coding region is deleted in the targeted *Pitx3* locus (Zhao et al., 2004). In contrast, the entire coding sequence remains intact in *aphakia* mice, which have 1±5% of the wild type level of *Pitx3* transcript from E11 to newborn (Rieger et al., 2001).

8.7. *Pitx3* null as a model for Parkinson's Disease

Parkinson's disease (PD) is characterised by the progressive loss of mDA substantia nigra neurons resulting in the depletion of dopamine in the striatum. Thus

PD affects the motor control system and the symptoms include tremor at rest, postural imbalance, slowness of movement, difficulty in initiating movement and rigidity (Lotharius and Brundin, 2002). The specific aetiology of PD is unclear, it seems that both genetic and environmental factors are involved. Rare familial cases of PD (0.5% of cases) have been linked to mutations in some genes such as Parkin, synuclein and ubiquitin-C-hydrolase-L1 (UCHL1). On the other hand, a range of factors including environmental toxins, oxidative stress and mitochondrial dysfunction have been implicated as non-genetic pathogenic mechanisms (Lotharius and Brundin, 2002). PD affects around 1% of the population over 65 years old and current treatments are mainly drug-based, which often have undesirable side effects (Klockgether, 2004). Therefore, there is much interest in animal models of PD which can be used to study the pathogenesis and develop treatments for this disease.

Ideally, an animal model for PD would have a full complement of mDA neurons at birth and the SNc mDA neurons would be selectively lost later in life. The loss of SNc mDA neurons should be at least 50-70%, the number which results in clinical symptoms in humans. In addition, the animal should display motor deficits similar to the main symptoms of PD. Current animal models of PD include the toxin-induced models using 6-hydroxydopamine, rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to ablate substantia nigra mDA neurons (Beal, 2001). More recently transgenic mice models with mutations in or overexpression of α-*synuclein* and *parkin* genes have been developed (Beal, 2001). At present the MPTP neurotoxicity model is the best of these animal models as it selectively targets the substantia nigra neurons and reproduces all the clinical signs of PD in primates. The main problem with this model is that it is an acute process rather than a slow progressive loss of mDA neurons as in PD. Furthermore, toxin-induced models of PD are not ideal due to the labour intensive toxin injections and the need to assess the degree of the lesion in each case. Thus genetic models of PD would be of great use for standardised high throughput screening of novel therapies for PD.

The specific loss of SNc mDA neurons in *Pitx3* null mice makes them a potential genetic animal model for PD. Although the behaviour of the *Pitx3* null mice has not been studied it is thought that it will be similar to that of *aphakia* mice. However, reports on the *aphakia* behavioural phenotype are inconsistent, in one study *aphakia* mice showed around 50-70% reduction in ambulatory and stereotypic activities and spent more time resting compared to wild type (Van Den Munckhof et

al., 2003). In contrast, Nunes *et al*. found that general locomotor behaviour appeared to be higher in *aphakia* as compared to wild type (Nunes et al., 2003). Another study stated that *Aphakia* mice had no altered posture, walking pattern or tremor, but they did have lower overall activity in an open field test and increased activity in a climbing test as compared to wild type (Smidt et al., 2004). A more recent study which focussed particularly on the behavioural phenotype of *aphakia* mice reported that there was no impairment in overall spontaneous locomotor activity (Hwang et al., 2005). Yet tests that were sensitive to nigro-striatal impairments, such as the challenging beam test, spontaneous exploratory behaviour test and the pole test, revealed that the *aphakia* mice displayed specific motor deficits. Importantly these impairments could be restored to wild type levels by treatment with the PD medication 3,4-dihydroxyphenylalanine (L-DOPA), which is a precursor of dopamine thus it compensates for the loss of dopamine from SNc neurons (Hwang et al., 2005). Further support that *aphakia* mice represent a valid model for PD is provided by their display of denervation supersensitivity of the striatum, which is an altered responsiveness of striatal neurons to dopamine. This occurs in PD patients and animal models of PD and can be reflected by the L-DOPA-mediated induction of the immediate early gene *c-fos* in the striatum (Hwang et al., 2005).

In *Pitx3* null mice (Ch. 6.2) and aphakia mice (Van Den Munckhof et al., 2003) SNc cells appear to die via apoptosis, however, the mode of cell death in PD remains controversial. Some studies have shown evidence for apoptotic cell death such as nick-end labelling (Mochizuki et al., 1996), DNA cleavage and chromatin clumping (Tatton et al., 1998) in SNc cells from brains of PD patients. Besides morphological evidence, factors involved in apoptotic pathways have been identified in SNc cells of PD patients. For example, activated caspase-3, a key player in mediating apoptosis (Hartmann et al., 2000) and activated caspase-8, a proximal effector protein of the tumor necrosis factor receptor family death pathway (Hartmann et al., 2001), have been found in more SNc cells of PD patients compared to control. However, other studies have found no evidence of apoptotic cell morphology such as nick-end labelling and no sign of up-regulation of apoptosis associated genes such as Bcl-x, Bcl-2, Bax and activated caspase-3 (Jellinger, 2000; Kosel et al., 1997).

In comparison to other existing transgenic models of PD, such as α*-synuclein* and *parkin* gene disruptions, the *Pitx3* null mice seems to be the most favourable due to the SNc specific loss of mDA neurons. Although, the rate of mDA neuron loss and

the presumed behavioural phenotype of the *Pitx3* null mice do not correlate well with PD. The lack of a severe behavioural phenotype in *Pitx3* null mice could be due to compensatory mechanisms as a result of the early developmental loss of the SNc neurons. Therefore, a model in which Pitx3 was conditionally knocked-out at a later stage in life may represent a better model of PD.

8.8. Concluding remarks

The work in this thesis has revealed that Pitx3 is specifically required for SNc mDA neuron survival and regulation of TH expression. Furthermore, this study has uncovered that during embryonic development there are two ontogenetically distinct groups of mDA neurons based on their expression of Pitx3 and TH. However, the picture of mDA neuron specification and development remains far from complete. Clearly there are many factors involved in mDA neuron specification and development that are yet to be uncovered. Future studies also need to address the relationships and interactions between the factors involved in the regulation of mDA neuron development and survival. Acquiring this knowledge may lead to a greater understanding of the pathogenesis of these neurons in human disease and aid in the realisation of the goal to derive pure populations of mDA neurons, possibly SNc-like mDA neurons, from ES cells.

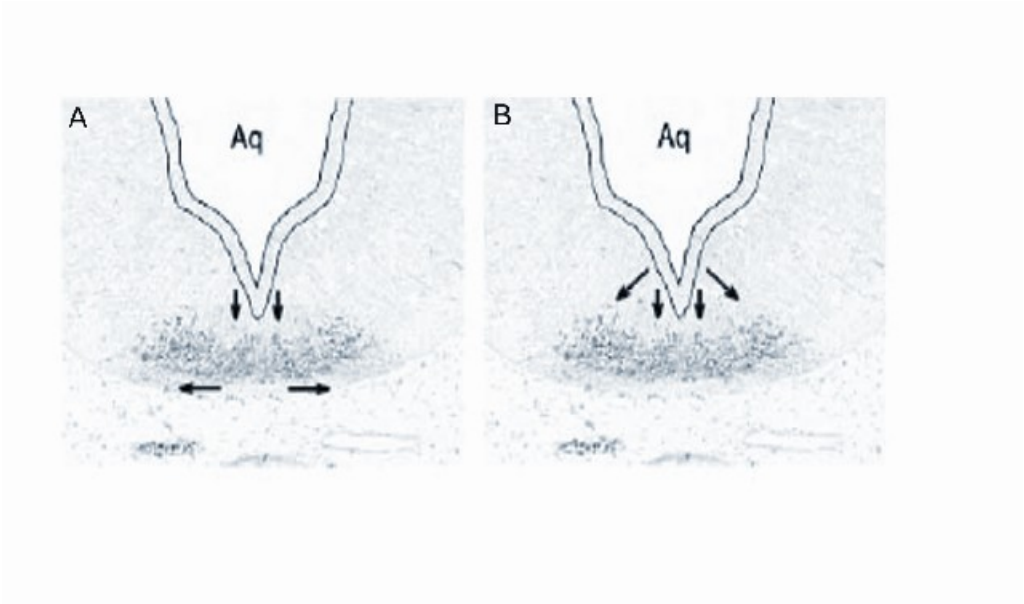

Figure 8.1. Models of mDA neuron migration.

Coronal sections of E12.5 midbrain with TH immunostaining are shown with the two proposed models of mDA neuron migration represented by arrows from the neuroepithelium which is indicated by a double line (A and B). Model A shows vertical migration from the most medial and ventral part of the neuroepithelium followed by lateral migration. This model is also known as the ‘inverted fountain’ model. Model B shows a pattern of radial migration from the medial and ventral one third of the neuroepithelium. Adapted from (Smidt et al., 2004).

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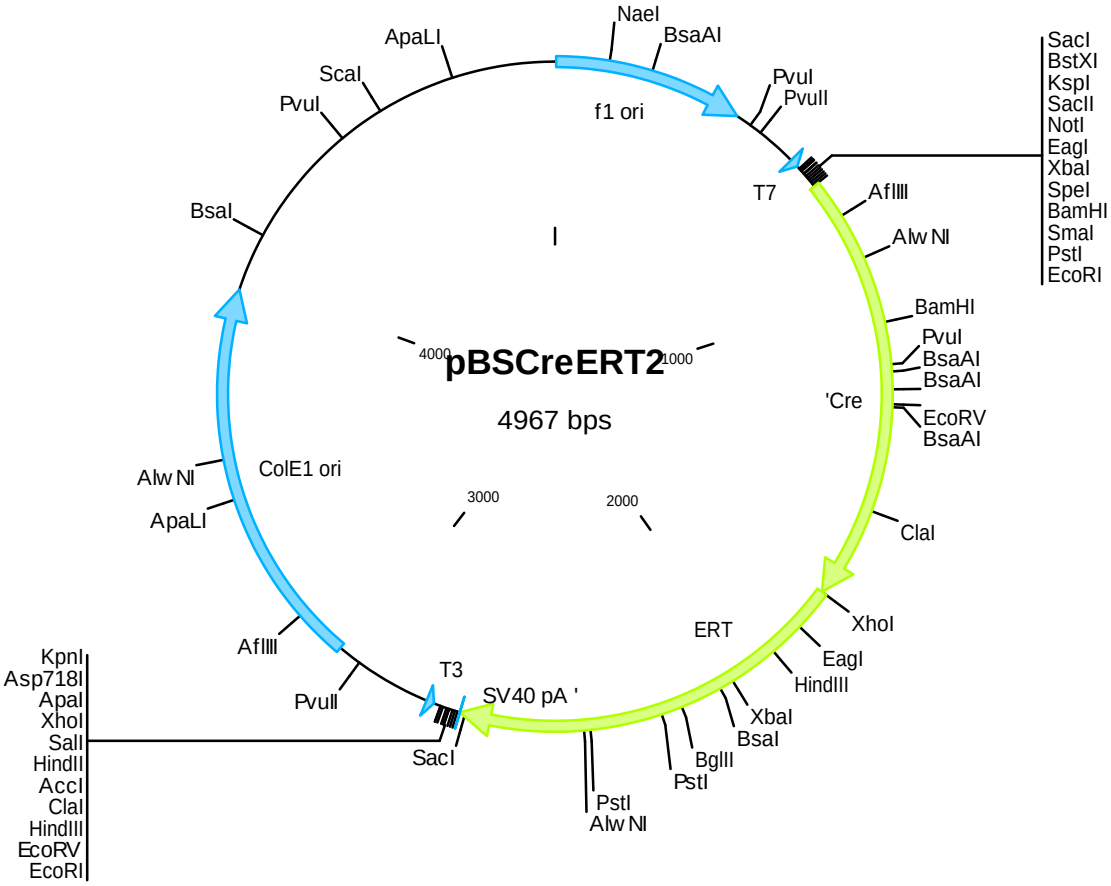
Appendix 1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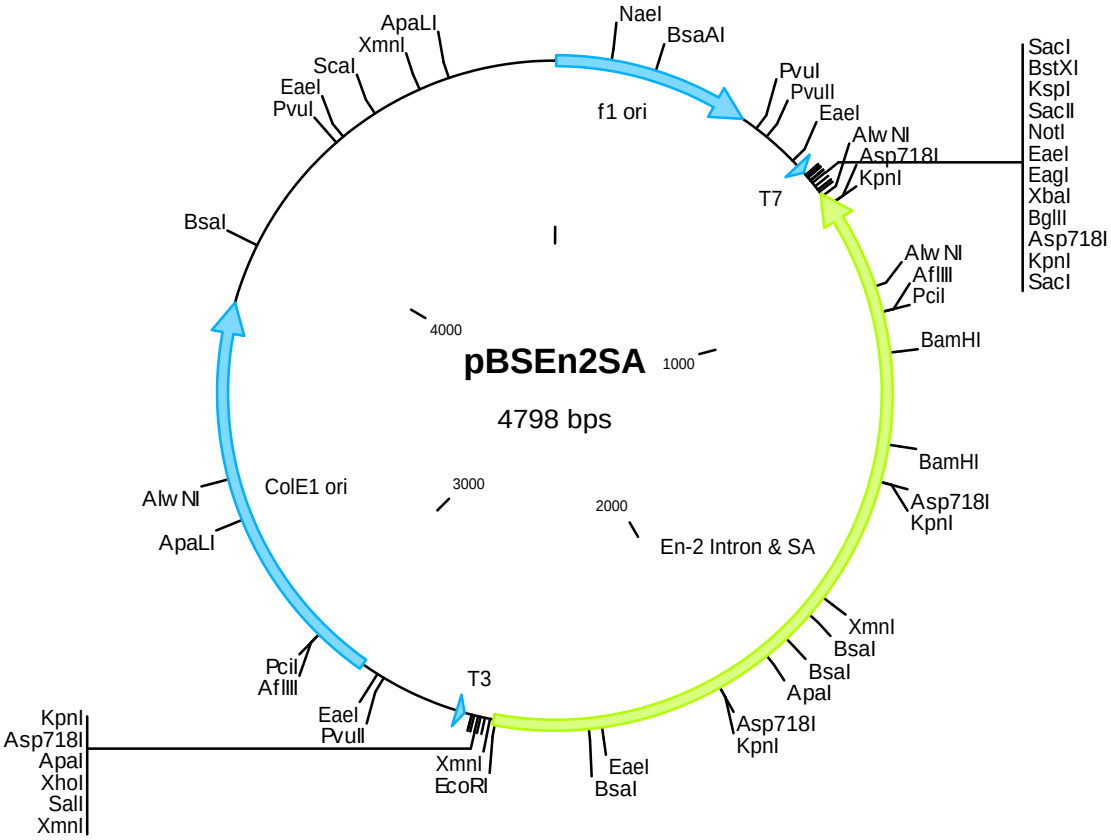
Appendix 2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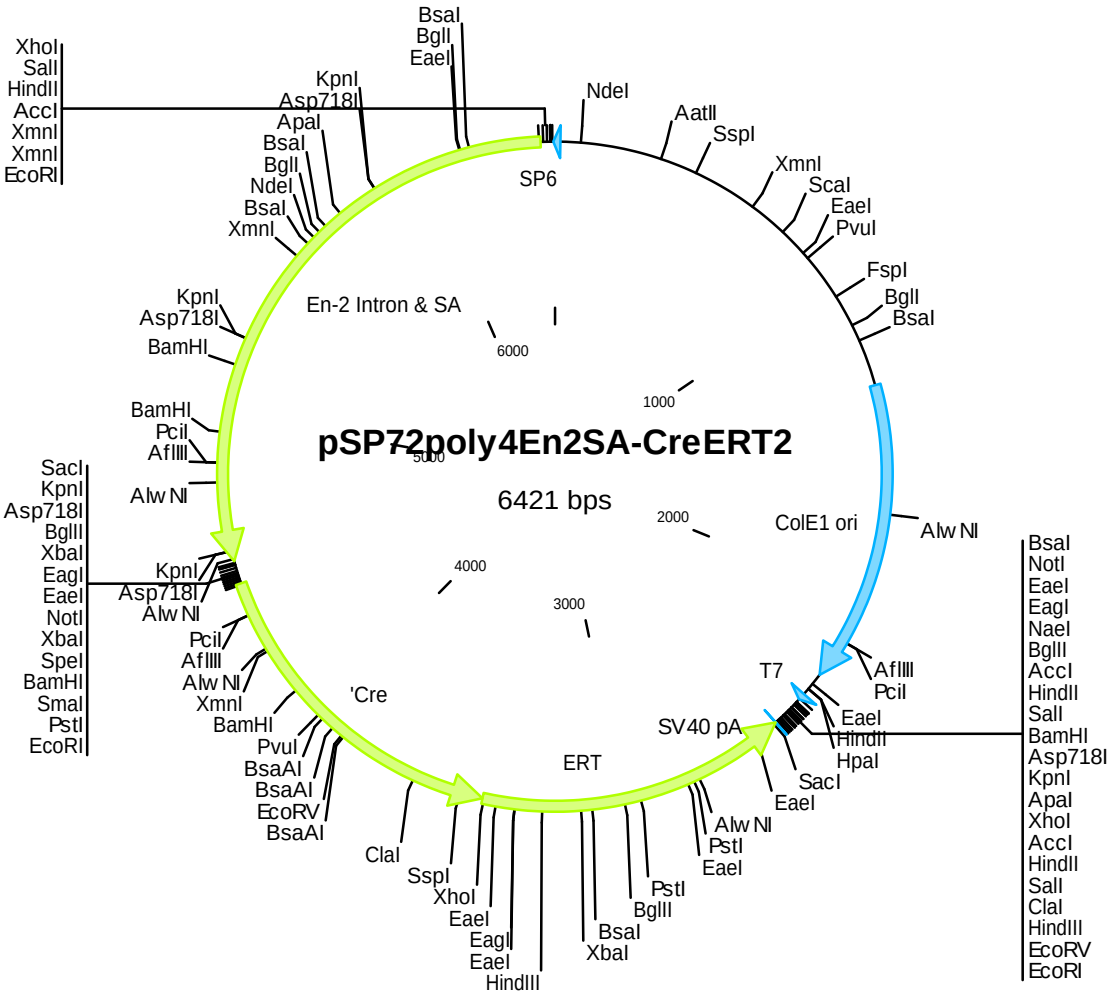
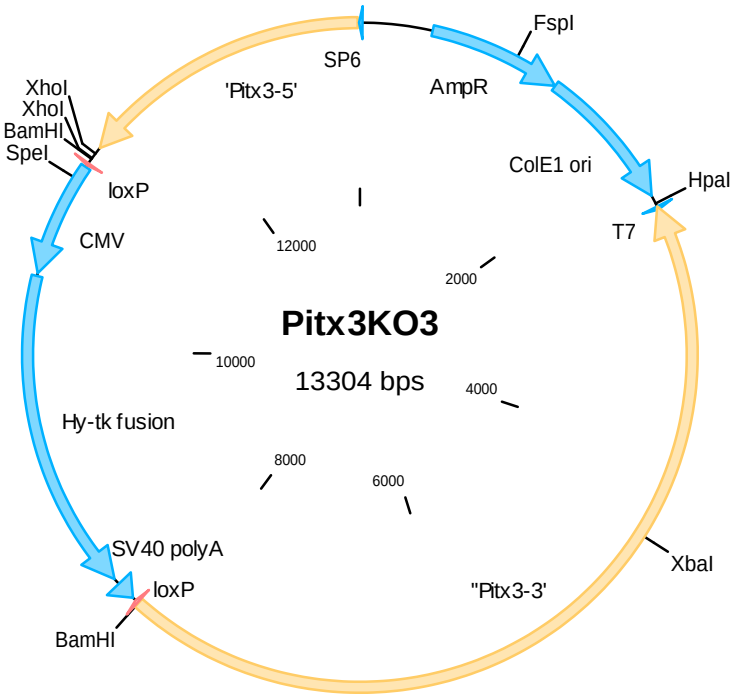
SalI
XmnI

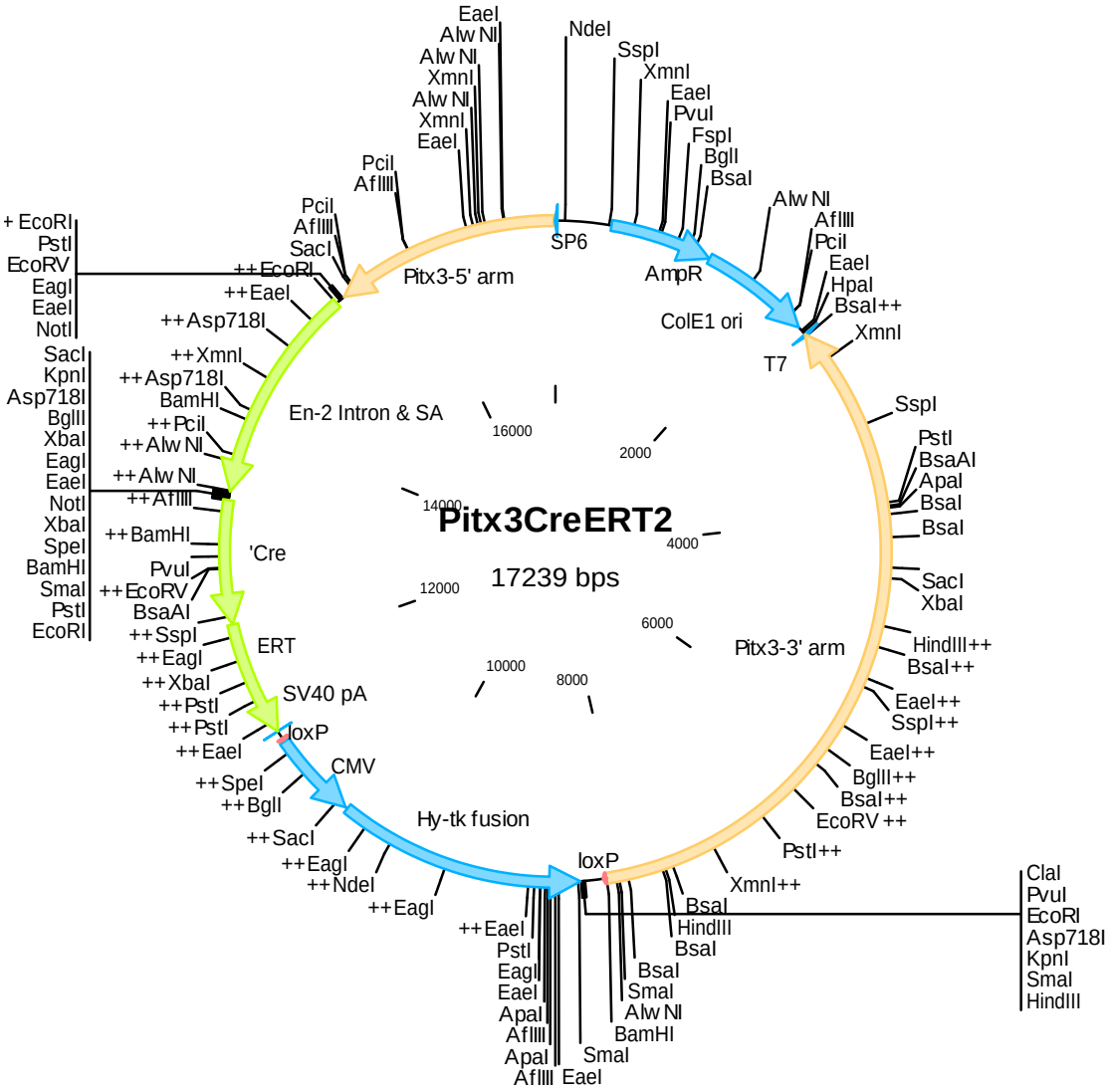
Appendix 3


pSP72poly4En2SA-CreERT2
6421 bps
SP6
En-2 Intron & SA
'Cre
ERT
ColE1 ori
T7
SV40 pA
1000
2000
3000
4000
5000
6000
BsaI
BglI
EaeI
NdeI
AatII
SspI
XmnI
ScaI
EaeI
PvuI
FspI
BglI
BsaI
AlwNI
AflIII
PciI
EaeI
HindII
HpaI
SacI
EaeI
AlwNI
PstI
EaeI
PstI
BglII
BsaI
XbaI
ClaI
SspI
XhoI
EaeI
EagI
EaeI
HindIII
BsaAI
BsaAI
EcoRV
BsaAI
PvuI
BamHI
XmnI
AlwNI
PciI
AflIII
AlwNI
KpnI
Asp718I
AlwNI
BamHI
PciI
AflIII
AlwNI
KpnI
Asp718I
BamHI
KpnI
Asp718I
BsaI
NdeI
BglI
BsaI
XmnI
KpnI
Asp718I
ApaI
XhoI
SalI
HindII
AccI
XmnI
XmnI
EcoRI
SacI
KpnI
Asp718I
BglII
XbaI
EagI
EaeI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
BsaI
NotI
EaeI
EagI
NaeI
BglII
AccI
HindII
SalI
BamHI
Asp718I
KpnI
ApaI
XhoI
AccI
HindII
SalI
ClaI
HindIII
EcoRV
EcoRI

Appendix 4


FspI
SP6
'Pitx3-5'
AmpR
XhoI
XhoI
BamHI
SpeI
loxP
ColE1 ori
HpaI
T7
CMV
12000
2000
Pitx3KO3
10000
13304 bps
4000
Hy-tk fusion
8000
6000
SV40 polyA
XbaI
loxP
"Pitx3-3'
BamHI

Appendix 5


Pitx3CreERT2
17239 bps
2000
4000
6000
8000
10000
12000
14000
16000
Pitx3-5' arm
En-2 Intron & SA
'Cre
ERT
SV40 pA
loxP
CMV
Hy-tk fusion
loxP
Pitx3-3' arm
T7
ColE1 ori
AmpR
SP6
EaeI
AlwNI
NdeI
SspI
XmnI
PvuI
FspI
BglI
BsaI
PciI
AflIII
SacI
EcoRI
Asp718I
BamHI
PstI
EcoRV
EagI
NotI
XbaI
SpeI
SmaI
BsaAI
ApaI
HpaI
HindIII
ClaI
KpnI